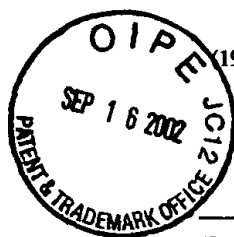


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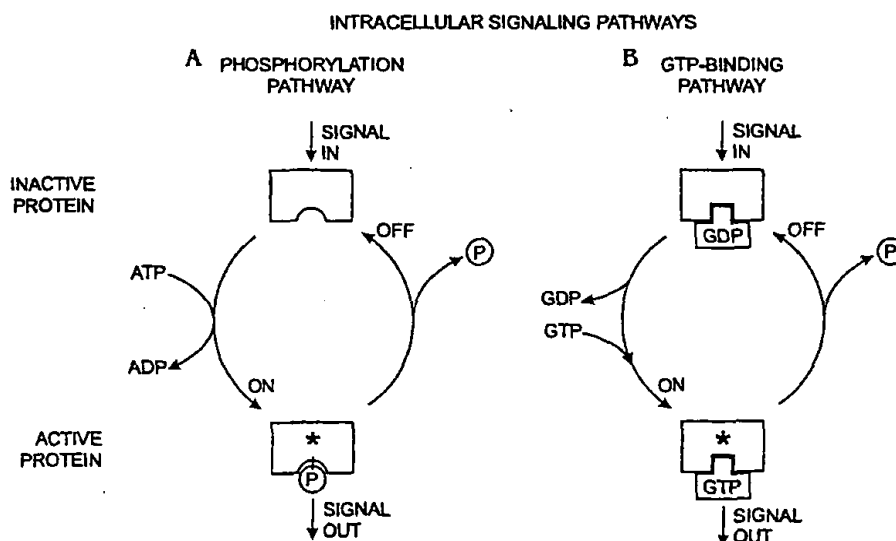
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(57) Abstract: Assays for detecting phosphorylation and dephosphorylation modifications of proteins, along with the presence, activity, and modulation of enzymes effecting these modifications, including kinases and phosphatases, respectively.

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5 June 9, 2000, entitled *Cell-Signaling Assays*, of inventors J. Richard Sportsman and Lawrence M. Kauvar.

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Field of the Invention

The invention relates to phosphorylation. More particularly, the invention relates to luminescence-based assays for detecting phosphorylation and dephosphorylation modifications of proteins, along with the presence, activity, and modulation of enzymes effecting these modifications, including kinases and phosphatases, respectively.

Background of the Invention

Cellular physiology may be regulated by a variety of mechanisms. In multicellular organisms, these mechanisms may involve cell-signaling pathways in which signal substances are released by one cell to influence the position, nature, and activity of other cells, for example, through intracellular phosphorylation and dephosphorylation reactions.

Figure 1 is a schematic view of a representative cell-signaling pathway 100. Here, signaling cells 102 produce signal substances 104_{a,b} that interact with target cells 106 to effect a response in the target cells. These responses may be short term, such as glycogen breakdown or muscle contraction, among others. These responses also may be long term, such as growth, differentiation, reproduction, and/or apoptosis, among others. Generally, these responses are brought about by increasing, decreasing, or maintaining enzyme activity in the target cells.

Signaling cells 102 are cells capable of producing a signal (substance) that can effect a specific response in another (target) cell. The signaling cells may be components of an endocrine, paracrine, or nervous system. The endocrine system is an organism-wide control system that regulates body function using hormones released by endocrine organs into the bloodstream. The endocrine organs include the pituitary gland, thyroid gland, parathyroid glands, adrenal glands, thymus gland, pineal body, pancreas, ovaries, testes, and kidneys. The paracrine system is a local control system that regulates nearby cells using local mediators released into the extracellular medium. The

nervous system is a specialized control system that regulates specific cells using electrical impulses and neurotransmitters.

Signal substances 104_{a,b} are substances through which a signaling cell may communicate with target cells, evoking a specific response. Signal substances may act as hormones, local mediators, and/or neurotransmitters, among others. Signal substances may take the form of proteins, small peptides, amino acids, nucleotides, steroids (e.g., cortisol, steroid sex hormones, vitamin D), retinoids, fatty acid derivatives, and dissolved gases (e.g., nitric oxide (NO) and carbon monoxide (CO)), among others.

Target cells 106 are cells capable of responding to a specific signal substance produced by a signaling cell. The ability to respond may depend on the cell and on the signal substance. For example, the signal substance thyroxine from the thyroid gland may evoke a response in nearly all cells, whereas the signal substance progesterone from the ovary may evoke a response only in specific cells in the lining of the uterus. The target response may include kinase activity, GTP binding, and/or cyclic nucleotide production.

The ability of a cell to respond to a given signal substance generally is determined by whether the cell includes a receptor for the signal substance. Here, a receptor is any molecule or supramolecular assembly capable of specifically binding a signal substance and initiating a response in a target cell. Representative receptors include cell-surface receptors 110 located on the surface of the target cell and intracellular receptors 112 located within the cytosol 114 or nucleus 116 of the target cell.

The nature of the response initiated by binding of a signal substance is determined by the intracellular machinery to which the receptor is operatively coupled. For example, binding of the neurotransmitter acetylcholine to identical receptors in heart muscle cells and secretory cells causes muscle relaxation in the heart muscle cells and secretion in the secretory cells, due to differences in the associated intracellular machinery.

The remainder of this section examines (1) the receptor mechanisms that cells use to bind signal substances and to communicate this binding to the cell interior, (2) the intracellular pathways that cells use for regulation, and (3) the effects of errors in cell-signaling pathways.

5 **1. Receptor Mechanisms**

Target cells generally have receptors capable of specifically binding specific signal substances, including cell-surface receptors and/or intracellular receptors, as described above. Cell-surface receptors are more common and include (A) G-protein-linked receptors, (B) enzyme-linked receptors, and (C)
10 ion-channel-linked receptors. These receptors typically bind large and/or water-soluble signal substances, such as many peptide hormones. Intracellular receptors are less common and include (A) guanylyl cyclase and (B) ligand-activated gene regulatory proteins. These receptors typically bind small and/or water-insoluble signal substances, such as steroid hormones, thyroid hormones,
15 retinoids, vitamin D, and NO.

Figure 2 is a schematic view of a representative G-protein-linked cell-surface receptor mechanism 130 that includes a receptor protein 132, a G-protein 134, and a target protein 136. These proteins may be positioned on or within the plasma membrane 138 of a target cell. In use, a specific signal
20 substance 140 binds to a signal-substance binding site 142 on the extracellular side 144 of the receptor protein and thereby creates, exposes, or otherwise activates (*) a G-protein binding site 146 on the intracellular side 148 of the receptor protein. The G-protein then binds to the G-protein binding site on the receptor protein and thereby creates, exposes, or otherwise activates (*)
25 a target-protein binding site 150 on the G-protein. The G-protein then dissociates from the receptor protein, binds (via the target-protein binding site) to the target protein, and activates (*) the target protein. Activation and deactivation of the G-protein may involve binding of a guanosine triphosphate (GTP) molecule and dephosphorylation of the GTP molecule, respectively. The

receptor protein may belong to a large superfamily of homologous, seven-pass transmembrane proteins. These seven-pass proteins consist of a single polypeptide chain that crosses the membrane seven times, with an extracellular signal-substance binding portion and an intracellular catalytic portion. The G-protein may be trimeric, consisting of three polypeptide chains-- α , β , and γ --that associate and dissociate during signaling. The target protein may consist of an enzyme or ion channel, among others. In particular, the target protein may be an enzyme that modulates the presence or activity of second messengers within the cell. These second messengers (also known as intracellular messengers or intracellular mediators) may bind allosterically to specific cellular proteins to alter their conformation and hence their activity. These second messengers include adenosine 3',5'-cyclic monophosphate (cAMP) and calcium (Ca^{2+}).

In the cAMP pathway, the target protein may be adenylyl cyclase (also known as adenylate cyclase), and the G-protein may be a stimulatory G-protein (G_s) that activates the adenylyl cyclase to make cAMP, or an inhibitory G protein (G_i) that inhibits the adenylyl cyclase to prevent it from making cAMP. The cAMP produced by the adenylyl cyclase activates cAMP-dependent protein kinase (A-kinase), which is a serine/threonine kinase that in turn activates or inhibits other enzymes to effect a physiological response. For example, in connection with glycogen metabolism, A-kinase may inhibit glycogen synthase to shut down glycogen synthesis, and simultaneously activate phosphorylase kinase that in turn activates glycogen phosphorylase to break down glycogen. A variety of signal substances use cAMP as a second messenger, including calcitonin, chorionic gonadotropin, corticotropin, epinephrine, follicle-stimulating hormone, glucagon, luteinizing hormone, lipotropin, melanocyte-stimulating hormone, norepinephrine, parathyroid hormone (PTH), thyroid-stimulating hormone, and vasopressin. The level of

cAMP is reduced by phosphodiesterases, and the activity of kinases is reversed by phosphatases, as described below.

In the Ca^{2+} pathway, the target protein may be a phospholipase with specificity for a phosphoinositide (i.e., inositol phospholipid), and the G-protein may be G_q , which activates the phospholipase to cleave the phosphoinositide to produce an intermediate that releases Ca^{2+} from the endoplasmic reticulum. For example, the phospholipase phosphoinositide-specific phospholipase C (phospholipase C- β) cleaves the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the second messengers inositol triphosphate (IP_3) and diacylglycerol. The inositol triphosphate is water soluble and diffuses to the endoplasmic reticulum (ER), where it releases Ca^{2+} from the ER by binding to IP_3 -gated Ca^{2+} -release channels in the ER membrane. The diacylglycerol is membrane bound and may be cleaved to form the second messenger arachidonic acid or may activate the Ca^{2+} -dependent serine/threonine kinase protein kinase C that in turn activates or inhibits other enzymes to effect a response. A variety of signal substances use Ca^{2+} as a second messenger, including acetylcholine, antigen, thrombin, and vasopressin.

Figure 3 is a schematic view of a representative enzyme-linked cell-surface receptor mechanism 170 that includes a receptor protein 172 positioned across the plasma membrane 174 of a target cell. The receptor protein includes a signal-substance binding site 176 on the extracellular side 178 of the membrane and a catalytic portion 180 on the intracellular side 182 of the membrane. (In some cases, the catalytic portion of the receptor may be replaced or augmented by a separate enzyme directly associated with the receptor protein.) In use, a specific signal substance 184 binds to the signal-substance binding site, initiating a series of events (such as dimerization and concomitant autophosphorylation of the receptor proteins) that activates (*) the catalytic portion of the receptor. The receptor protein may belong to one of at least five classes of single-pass transmembrane proteins: (A) receptor guanylyl

cyclases, which catalyze the production of guanosine 3',5'-cyclic monophosphate (cGMP) in the cytosol; (B) receptor tyrosine kinases, which phosphorylate specific tyrosine residues on some intracellular proteins, (C) tyrosine-kinase-associated receptors, which associate with proteins that phosphorylate specific tyrosine residues on some intracellular proteins; (D) receptor tyrosine phosphatases, which dephosphorylate specific tyrosine residues on some intracellular proteins, and (E) receptor serine/threonine kinases, which phosphorylate specific serine or threonine residues on some intracellular proteins. Some of these receptors are described below in more detail.

The signal substance also may bind to intracellular receptors, such as guanylyl cyclase. This enzyme produces cGMP from GTP, which then acts as a second messenger much like cAMP. As described above, cGMP also may be produced by enzyme-linked cell-surface receptors. cGMP is present in most tissues at levels 1/10 to 1/100 those of cAMP. A variety of compounds increase cGMP levels in cells, including (1) the hormones acetylcholine, insulin, and oxytocin, (2) the guanylate cyclase stimulators (and vasodilators) nitroprusside, nitroglycerin, sodium nitrate, and nitric oxide, (3) chemicals such as serotonin and histamine, and (4) peptides such as atrial natriuretic peptide (ANP) that relax smooth muscle.

2. Intracellular Signaling Pathways

Target cells may have intracellular signaling pathways capable of specifically binding signal substances, including cell-surface receptors and intracellular receptors, as described above. These pathways may include (1) a phosphorylation pathway involving ATP/ADP, and (2) a GTP-binding pathway involving GTP/GDP.

Figure 4A is a schematic view of a representative phosphorylation pathway. Phosphorylation is the predominant mechanism used to regulate protein activity in eucaryotic cells. In phosphorylation, a phosphate group (P) is

reversibly attached to the side chain of an amino acid in a protein. The attached phosphate group may cause structural changes in the protein, for example, due to electrostatic interactions between the negative charges on the phosphate group and positive charges on the side chains of nearby amino acids. These structural changes may affect the activity of the phosphorylated protein, enhancing or inhibiting its function.

Specialized enzymes control phosphorylation in cells. In particular, protein kinase enzymes transfer phosphate groups to proteins, and protein phosphatase enzymes remove phosphate groups from proteins. Protein kinases and protein phosphatases are found in great variety in eucaryotic cells: a single cell may contain more than 100 different kinases, and one percent of genes may code for kinases.

There are two major categories of kinases: (1) serine/threonine (S/T) kinases, and (2) tyrosine kinases. The S/T kinases function by selectively phosphorylating serine and threonine side chains on substrate proteins or peptides. These kinases include cyclic AMP-dependent kinase (A-kinase), cyclic GMP-dependent kinase (G-kinase), protein kinase C (C-kinase), Ca^{2+} -calmodulin-dependent kinase (CaM-kinase), phosphorylase kinase, MAP kinase, and TGF- β receptor, among others. The S/T kinases are predominantly cytosolic. The tyrosine kinases function by selectively phosphorylating tyrosine side chains on substrate proteins or peptides. These kinases include the receptor kinases for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin, insulinlike growth factor-1 (IGF-1), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and macrophage colony stimulating factor (M-CSF). These kinases also include the nonreceptor kinases associated with the tyrosine-kinase-associated receptors, such as the Src family (Src, Yes, Fgr, Fyn, Lck, Lyn, Hck, and Blk) and Janus family (JAK1, JAK2, and Tyk2) kinases. The tyrosine kinases are predominantly membrane bound.

A few kinases function by selectively phosphorylating threonine and tyrosine side chains on substrate proteins or peptides. These kinases include the mitogen-activated protein (MAP) kinase-kinase.

Figure 4B is a schematic of a representative GTP-binding pathway. The GTP-binding pathway generally resembles the phosphorylation pathway in that each pathway involves transfer of a phosphate group to a protein. However, in the GTP-binding pathway, the protein gains a phosphate group by exchanging a bound GDP for a bound GTP, whereas in the phosphorylation pathway, the protein gains a phosphate group by covalent addition of the phosphate group to a serine, threonine, or tyrosine by a kinase enzyme. The binding of a GTP to a GTP-binding protein may cause structural changes in the protein that in turn affect the activity of the protein. Examples of GTP-binding proteins include the trimeric G-proteins described above and the Ras superfamily of monomeric GTPases. The Ras proteins are activated by release of bound GDP and binding of GTP stimulated by guanine-nucleotide releasing proteins (GNRPs). The Ras proteins are inactivated by hydrolysis of the bound GTP by GTPase-activating proteins (GAPs).

A physiological response may require stimulation by only a single type of signal substance, or may require stimulation by two or more types of signal substances. The latter mechanism permits finer tuning of the physiological response through signal integration. For example, a protein may be activated only by phosphorylation by two different kinases, themselves activated by binding of two different signal substances to two different receptors. Alternatively, a protein may be activated only by concurrent phosphorylation and GTP binding, or by binding of two subunits whose binding is contingent on phosphorylation by separately activated kinases.

3. Effects of Errors

Errors in the signal transduction and regulation pathways described above can cause cancer and other diseases. Indeed, a primary cause of cancer is

a mutation that makes a stimulatory gene product hyperactive, converting a proto-oncogene into an oncogene. The primary classes of known proto-oncogenes include the following cell-signaling proteins: (1) growth-factor receptors acting via tyrosine kinases, (2) GTP binding proteins, (3) 5 membrane/cytoskeleton-associated tyrosine kinases, (4) cytoplasmic tyrosine kinases, (5) steroid-type growth-factor receptors, and (6) S/T kinases. Consequently, cell-signaling proteins have become important subjects of research and drug development.

Assays that determine the presence and activity of cell-signaling 10 components are important tools for high-throughput screening laboratories. Unfortunately, current assays have a number of shortcomings. For example, the presence and activity of kinases can be determined using assays capable of detecting phosphorylated amino acids. In a standard kinase assay, radioactive ATP and an appropriate protein substrate are added to a sample. If the sample 15 includes kinases, radioactive phosphate groups will be transferred from the radioactive ATP to the protein substrate. The protein substrate and radioactive ATP can be separated, and the presence and activity of kinases determined by assaying the amount of radioactive protein substrate. Unfortunately, this assay involves radioactivity, presenting a short-term safety hazard for the assay 20 operator and a long-term storage and disposal problem. Moreover, the assay is heterogeneous, requiring separation of components for analysis. Significantly, assays for other cell-signaling components may have similar shortcomings, as well as slow time courses and unstable endpoints that require precise timing of assay readouts. Thus, there is a need for improved assays for detecting the 25 presence and activity of cell-signaling components.

Summary of the Invention

The invention provides luminescence-based assays for detecting phosphorylation and dephosphorylation modifications of proteins, along with

the presence, activity, and modulation of enzymes effecting these modifications, such as kinases and phosphatases, respectively.

Brief Description of the Drawings

Figure 1 is a schematic view of a cell-signaling pathway.

5 Figure 2 is a schematic view of a G-protein-linked cell-surface receptor mechanism that includes a receptor protein, a G-protein, and a target protein, all associated with the plasma membrane of a target cell.

 Figure 3 is a schematic view of an enzyme-linked cell-surface receptor mechanism that includes a receptor protein positioned across the plasma
10 membrane of a target cell.

 Figure 4 is a schematic view of two common intracellular signaling pathways: (A) a phosphorylation pathway involving ATP/ADP, and (B) a GTP-binding pathway involving GTP/GDP.

 Figure 5 is a schematic view of several assays provided by the invention.

15 Figure 6 is a diagrammatic representation of one of the assays of Figure 5.

 Figure 7 shows the dependence of luminescence polarization on the concentration of antibody available to react with a labeled tracer peptide.

 Figure 8 shows the results of an assay in which unlabeled "modified
20 substrate" is able to displace luminescently labeled tracer in binding to antibodies that specifically bind both, in a concentration-dependent manner.

 Figure 9 shows a dose-dependent competition assay with labeled tracer, in analogy with Figure 8.

 Figure 10 shows a competition assay with a modified substrate
25 generated by the action of the insulin receptor cytoplasmic kinase domain (IR-CKD), plotted as a function of μL catalysis mixture [CKD+buffer+ATP+substrate peptide] added to constant amount of antibody and tracer.

Figure 11 shows the use of the assay of Figure 10 to assess kinase activity of IR-CKD, plotted as a function of time for a constant amount of catalysis mixture.

Figure 12 shows the use of the assay of Figure 10 to assess inhibitor
5 activity at a fixed time.

Figure 13 shows the use of the assay of Figure 10 to assess agonist activity with respect to IR-CKD at a fixed time.

Figure 14 shows the use of the invention assay for a CKD different than the CKD of the insulin receptor.

10 Figure 15 is an HPLC chromatographic trace of fragments of IR-CKD.

Figure 16 shows the use of the competition assay of the invention to map the location of phosphorylated tyrosine in the separated peptides.

Figure 17 is a schematic view of a tyrosine kinase assay.

Figure 18 is a graph showing competition curves for a tyrosine kinase
15 assay in which a mixture of tracer and antibody is added to a simulated enzyme product (o-phospho-L-tyrosine).

Figure 19 is a graph showing competition curves for a tyrosine kinase assay in which a mixture of EDTA, tracer, and antibody is added to the simulated enzyme product of Figure 18.

20 Figure 20 is a graph of polarization versus phosphotyrosine competitor concentration in 96-well and 1536-well microplates, showing how luminescence polarization may be used in a homogeneous immunoassay format to measure inhibition of enzyme activity.

Figure 21 is a graph of polarization versus phosphotyrosine competitor
25 for the assay of Figure 19, determined for sample volumes of 40, 60, 80, and 100 μ L concentration in 384-well microplates.

Figure 22 shows the effects of incubating 10 nM TK-1 tracer with different concentrations of MM-GA

Figure 23 shows a dose-response curve for ATP, with 10 nM TK-1 tracer and 1.6 nM (estimated) MM-Ga.

Figure 24 shows a dose-response curve for TK-1 calibrator, with 10 nM TK-1 tracer and 1.6 nM (estimated) MM-Ga.

5 Figure 25 shows binding of a fluorescein-labeled mono-phosphoserine peptide STK-1 tracer to various concentrations of MM-Ga.

Figure 26 shows the effects of HEPES-based buffers on the MM-Ga/phosphopeptide interaction.

Figure 27 shows an endpoint assay of PKA activity with MM-Ga.

10 Figure 28 shows a time-course assay of PKA activity with MM-Ga performed under the reaction conditions of Figure 27.

Figure 29 shows a time-course assay of PKA activity with a Ga^{3+} -coated plate.

15 Figure 30 shows polarization calibration curves for TK-1 with BODIPY-TMR tracer, for (A) 500 msec and (B) 200 msec integration times.

Detailed Description

The invention provides luminescence-based assays for detecting phosphorylation and dephosphorylation modifications of proteins, along with the presence, activity, and modulation of enzymes effecting these modifications, including kinases and phosphatases, respectively. These assays may include luminescence polarization and luminescence energy transfer assays, among others. These assays also may include peptide substrates and binding partners specific to phosphorylated or unphosphorylated versions of the substrates, or phosphorylated substrates in general.

25 Luminescence assays have been described. In a typical format, the ability of an analyte to compete for a specific binding partner with a luminescently labeled form of an analyte or analog is measured in a heterogeneous assay by determining, for example, the level of the luminescent competitor in a solution or attached to a solid support after the competitor and

the analyte are contacted with a member of a specific binding pair to which both bind, which member is coupled to the solid support. The amount of luminescent label bound to the solid support is inversely proportional to the concentration of the analyte, while the amount of luminescent label in the solution is directly proportional thereto.

Such an assay is possible in homogeneous form if the luminescence polarization or other measurable property of the luminescent label changes when it is bound to the opposing member of its specific binding pair. If the level of polarization, for example, is enhanced when the luminescent competitor is bound, the degree of polarization will be inversely correlated with the concentration of the tested-for analyte. See, for example, Jameson, D.M., et al., "Fluorescence Anisotropy Applied to Biomolecular Interactions," in Methods in Enzymology (1995) 246:283-300.

Luminescence assays also have been used to detect proteolytic activity where proteolysis liberates a luminescently labeled material. Separation of the liberated luminescent tracer thus measures the proteolytic activity of an enzyme. See U.S. Patent No. 5,235,039.

Recently, an abstract by Seethala, K. published in advance of the meeting of the Society for Biomolecular Screening, to be held September 22-25, 1997, reports the development of a "fluorescence polarization-competition immunoassay . . . in which the tyrosine kinase phosphorylated peptide/protein competes with luminescent phosphopeptide used as a tracer in a pY antibody immunocomplex." Here, pY represents phosphoryl tyrosine.

The invention provides assays generally applicable to determining the presence, activity, and/or modulation of proteins effective in making posttranslational modifications of synthesized protein, especially phosphorylation and dephosphorylation modifications at tyrosine, threonine, or serine residues. Figure 5 shows a few of these assays, in particular, luminescence-polarization-based (FP) assays. Here, S is a substrate containing

an amino acid such as tyrosine, threonine, or serine capable of being phosphorylated, pS is a phosphorylated version of the substrate, S* and pS* are luminescently labeled versions of S and pS, respectively, and the substantially rectangular box is a binding partner capable of recognizing pS and pS*, but not S or S*. Panel A shows a direct kinase assay, in which S* is phosphorylated by a kinase to yield pS*, which binds to the binding partner, leading to an increase in polarization. Panel B shows an (indirect) competition kinase assay, in which S (typically present in excess of pS*) is phosphorylated by a kinase to yield pS, which competes off pS* for binding to the binding partner, leading to a decrease in polarization. Panel C shows a direct phosphatase assay, in which pS* is dephosphorylated by a phosphatase to yield S*, which does not bind to the binding partner, leading to a decrease in polarization. Analogous assays can be constructed using a binding partner that recognizes S and S*, but not pS or pS*, namely, a direct assay for kinase activity and direct and (indirect) competition assays for phosphatase activity.

The invention also provides assays that can be performed in a homogeneous environment. The assays may involve competition of a luminescent tracer for an opposing member of a specific binding pair, such as an antibody. In these assays, the tracer competes with a modified form of a substrate where the modification may include any posttranslational modification of a protein. The concentration of the modified substrate may be used as an index to the activity of the enzyme catalyzing the modification, or as an index to an agonist with regard to that activity or an inhibitor with regard to that activity, or it may be used to map sites of modification following fragmentation of the protein by physical or enzymatic means. If the enzyme is associated with a receptor, the assay effectively measures the ability of a test compound to act as an agonist or antagonist of the receptor, modulating activity of the receptor. Thus, the assays of the invention provide a broad spectrum of assessment to evaluate metabolism, protein synthesis, and catalytic activities.

Thus, in one aspect, the invention is directed to a method to determine the concentration of a modified substrate which method comprises contacting a sample containing the modified substrate with a luminescent tracer, wherein the luminescent tracer and the modified substrate compete for binding to a substance, followed by measuring the luminescence polarization in the sample. When the tracer is bound to the substance, its luminescence polarization is changed from the value associated with the unbound form of the luminescent tracer.

The invention in its various aspects may include and/or involve among others one or more of the following: (1) analytes, (2) tracers, (3) specific binding partners, and (4) modulators. In most cases, the format of the assay will place constraints on the preferred relative concentrations of these components and on the preferred lifetime of the luminophore associated with the tracer. For example, in a competition assay, the concentration of binding partner is preferably at least about equal to the concentration of tracer (to permit stoichiometrically an appreciable fraction of the tracer to be bound), and the affinity of the binding partner for the tracer is preferably such that the dissociation coefficient K_d describing interaction between the binding partner and tracer is about equal to the concentration of the free binding partner (so that the fraction of bound tracer is not too close either to 0 or 1, to maximize sensitivity). Moreover, the lifetime of the luminophore associated with the tracer preferably is longer than the rotational correlation time of free tracer and shorter than the rotational correlation time of bound tracer, and, all else being equal, ideally equal to the geometric mean of the two rotational correlation times of free and bound tracer (to obtain the largest polarization change upon binding).

The "modified substrate" may be a posttranslationally modified protein or peptide, such as a phosphorylated peptide, and the sample may be an intact sample or a set of fragments thereof. The substance for which the tracer and

modified substrate compete is typically an antibody, but includes any specifically binding substance that results in a change in the luminescence polarization of the tracer.

In another aspect, the invention is directed to luminescent tracers used in specific embodiments of the invention, as well as cell lines that provide intracellular specific binding pair members useful in the assays.

The method of the invention takes advantage of the change in luminescence polarization of a luminescently labeled substance when the substance becomes bound to the opposing member of a specific binding pair of which the substance itself is a member. Typically, the substance is a peptide or protein.

By "opposing member of a specific binding pair" is meant a member or a partner of the pair that binds specifically but noncovalently to a subject material that is the other members or partners. Such terminology is familiar in a number of contexts. Thus, antigens and antibodies are opposing members of specific binding pairs, as are ligands and receptors, and as are avidin and biotin. Depending on the application, a specific binding partner may bind substantially exclusively only to a particular species, such as a phosphorylated peptide having a particular sequence as opposed to other sequences, or only to a particular class of species, such as phosphorylated peptide as opposed to an unphosphorylated peptide.

To function in a competition assay, both competitors must bind substantially to the opposing member of the specific binding pair. Thus, the competitors may be the analyte to be assayed and the analyte in luminescently labeled form, i.e., coupled to a luminescent material. Peptides are convenient analytes, as labeled forms can be prepared easily. Thus, the analyte itself is typically a peptide and especially a modified form of the peptide that can be distinguished through specific binding from the unmodified form. Thus, as used herein, "modified substrate" refers to a substrate for an enzyme-catalyzed

reaction that has been converted to product. Exemplified herein is an assay where the modified substrate is the phosphorylated form of an unmodified peptide, wherein antibodies can be prepared which bind specifically to the phosphorylated form.

5 In the illustrated embodiment, the tyrosine residue (Y) in the peptide is phosphorylated. However, other modifications to peptides and proteins are well known and can be detected by using such modified substrates as analytes as well. A summary of known posttranslational modifications of proteins and peptides is set forth in *Advances in Enzymology* (1993) 67:265-299, which is
10 incorporated herein by reference. For example, the N-terminus of various peptides can be modified by acylation (e.g., by formyl, acetyl, myristoyl, lauroyl, tetradeca (mono and di) enoyl, α -ketoacyl, and aminoacyl groups), and also can be derivatized with methyl, pyrrolidone carboxyl, gluconuryl, and glycosyl groups. Similar substitutions can take place at the nitrogen of lysine,
15 including lipoyl substitution, biotinyl, ubiquitinyl, phosphoryl, pyridoxyl, and glycosyl. Hydroxy groups also can be added to the lysine at the δ position, which can then be further derivatized. Arginine can be derivatized as the ADP-ribose derivative, alkyl derivative, and additional substitutions. A variety of acyl groups, specifically of fatty acids, can be reacted with the sulfhydryl group
20 of cysteine, along with coenzymes, prenylation, and the like. Tyrosine can be substituted at the hydroxyl group by a variety of substituents and can be halogenated as well. Modifications may be a result of enzymatic or nonenzymatic processes, for example, glycosylation of hemoglobin in diabetic patients.

25 Common groups that are phosphorylated include tyrosine, serine, and threonine.

 In the foregoing cases, antibodies can be made to the modified proteins that fail to react significantly to either the unmodified or modified form. Such antibodies are useful as the opposing members of the specific binding partner

for analyte. Thus, antibodies can be prepared by using the modified substrate as an immunogen, harvesting appropriate antibody producing cells and immortalizing them, and performing appropriate screening tests. The tests select immortalized cells that produce antibodies that bind to the modified
5 substrate, but discard those that also bind to the substrate itself. In this manner, a suitable opposing member of a specific binding pair can, in principle, be prepared with respect to any modified or unmodified substrate.

Figure 6 shows a diagrammatic representation of the overall aspects of the assay. Antibodies to phosphoryl tyrosine (pY) are illustrated as the
10 opposing member of the specific binding pair. As shown in flask A, a significant fraction of the luminescent tracer is bound to the specific antibodies, and the luminescence polarization output is high. In flask B, however, a kinase that phosphorylates the tyrosine residues on a substrate is introduced, and the modified substrate is thus present in flask B. The modified substrate displaces
15 the luminescent tracer from the antibodies and thus reduces the polarization of the sample.

In its simplest form, the assay can be constructed so as simply to measure the concentration of the analyte. The analyte can be any substance for which a luminescent tracer can be prepared by coupling a luminescent label to
20 it or a close analog, and for which an opposing member of a specific binding pair can be obtained which is competitively bound by the tracer and the analyte. Thus, any arbitrary substance can be used as the analyte in this assay. However, the assay of the invention is particularly useful when the analyte is a "modified substrate" that is produced by an enzyme-catalyzed reaction
25 converting a substrate to its modified form. Thus, if the analyte is a modified substrate formed through a catalyzed reaction, the assay may be designed so as to measure the activity of the catalyst and to screen for compounds that enhance or inhibit this activity. If the activity is associated with a receptor,

agonists and antagonists for the receptor may be measured by the assay of the invention.

Thus, by measuring the concentration of analyte as a function of time, or at a suitable preset time, the level of catalytic activity in its formation can be assessed. The effect on the time course of analyte concentration as produced by additional substances serves to indicate whether these substances are enhancers or inhibitors of this activity.

The luminescent label can be any of a number of dyes as long as the dye exhibits luminescence polarization when the substance to which it is conjugated is bound to the opposing member of the specific binding pair. Fluorescein is a typical fluorescing unit, which can be coupled, for example, through the corresponding isothiocyanate to a free amino group in the tracer substrate. Other luminescent compounds that are suitable include dansyl, rhodamine, and Texas Red.

To conduct the assay, a sample of the modified substrate or an analog representing it is first coupled to a luminescent label through standard linkage technology known in the art. The resulting labeled tracer is then titrated with the opposing member of its specific binding pair, which also is capable of binding to the modified substrate per se. The substrate for an enzyme catalyzed reaction, the enzyme, and any necessary cofactors are combined to form a catalysis mixture, which is added immediately or at fixed intervals to buffer containing the specific binding pair member and the luminescent tracer. As the enzyme catalyzes the conversion of the substrate to the modified form, the modified form is detected through competition with the luminescent tracer. The amounts of reagents are adjusted so as to provide distinction from background polarization and to obtain meaningful results. The amount of modified substrate under a variety of conditions of concentration of components or at various times is then measured by determining luminescence polarization.

Measurement of luminescence polarization is well known and can be conducted with standard laboratory equipment.

If the bound form of the tracer has a higher luminescence polarization than the tracer in free form, the luminescence polarization of the sample will
5 decrease as more modified substrate is formed. By measuring the luminescence polarization at various times, then, the rate of conversion of substrate to modified substrate can be determined directly. Alternatively, the level of enzyme can be measured by determining the luminescence polarization at a set time at a series of dilutions of the catalysis mixture containing the enzyme,
10 substrate, and cofactors. Accordingly, the level of enzyme that converts substrate to modified substrate can be determined, and the effect of any stimulator or inhibitor molecules on the activity of the enzyme can be assessed.

The following examples are intended to illustrate but not to limit the invention. They illustrate the application of the invention method to study the
15 features of the insulin receptor, to determine the kinase activity of the receptor, and more specifically of the cytoplasmic kinase only domain, and thereby to identify agonists and antagonists of the receptor. The insulin receptor contains two α chains and two β chains which are assembled at the cell membrane. The α chains are exposed on the outside of the cell and contain the insulin-binding
20 site. The β chains extend to the interior of the cell and contain a binding site for ATP, as well as tyrosine residues that are phosphorylated when the receptor is stimulated by insulin. Thus, the peptides not phosphorylated at the relevant tyrosine residues are the substrate for the autophosphorylation activity of the insulin receptor, and the pY form is the modified substrate.

25 A primary substrate for the kinase activity of the activated receptor, IRS-1, undergoes tyrosine phosphorylation as an early step in the insulin receptor signaling pathway, and it too could be assayed by the method of the invention.

Further, sites of phosphorylation within a protein can be mapped following digestion of the protein and HPLC separation of the fragments. Such a method could map a protein for the location of modification sites, and would include as first and second steps the digestion of a protein with a protease to
5 obtain fragments and then the separation of the fragments. The fragments are then treated with a luminescence polarization mixture comprising a tracer and an opposing specific binding pair member, where the tracer and any modified fragments compete with one another for binding to the opposing specific binding pair member. A detection of a change in luminescence polarization
10 would indicate a modified fragment.

The following examples are intended to illustrate without limitation various aspects of the invention.

Example 1

Effect of Binding on Luminescence Polarization

15 As the ultimate intention is to measure by pY content the modified substrate of the insulin receptor kinase, a phosphorylated peptide that mimics the portion of the substrate that will become phosphorylated by virtue of the kinase activity is used as the labeled tracer. This peptide may be relatively simple, and thus applicable to many kinases, or it may be a more specific
20 mimic of one particular kinase substrate. The phosphorylated form of the mimicking peptide will thus bind to anti-pY antibodies of greater or lesser specificity as will the modified substrate itself. To determine whether such a tracer would indeed exhibit enhanced luminescence polarization when bound to anti-pY antibodies, the following preliminary studies were conducted.

25 The peptide to be coupled to luminescent label was of the formula GEEGYMPMGK, where the tyrosine residue is chemically phosphorylated, and the C-terminal lysine is derivatized on the ϵ amino group with a biotin. The derivatization with biotin is irrelevant to the claimed assay. The tracer form of

this peptide was prepared by coupling fluorescein to the amino terminus by reaction with fluorescein isothiocyanate (FITC).

The luminescence polarization experiment was tested on two types of readers: an FPM-1 Fluorescence Polarization Analyzer, a single 12×75 mm tube reader (Jolley Consulting and Research, Inc.), and a FPM-2, a 96-well reader.

The single-tube reader is a more sensitive instrument, and can use less tracer. In the FPM-1, the tracer form of the peptide (GEEGYMPMGK) coupled with fluorescein to the amino terminus was used at 0.3 nM. To transfer this technology to multiple samples, the assay was transferred to a 96-well format. In doing so, the sensitivity of the instrument is less, and as a result more tracer is required to give adequate signal. We were able to see 7.5 and 15 nM of the tracer form of the peptide coupled with fluorescein. For the simpler tracer, FL-phosphotyramine, we had to use 35 nM of the tracer.

Phosphotyramine has been conjugated to fluorescein to give the tracer FL-phosphotyramine. When mixed with an anti-phosphotyrosine antibody, it can be competed off with N-Acetyl-O-Phosphotyramine. N-Acetyl-O-Phosphotyramine is used as the calibrator for the assay in this instance.

It also is necessary, of course, that the labeled tracer is effectively displaced when an unlabeled form of the phosphorylated peptide is added. Upon addition of 2 µg/mL of the unlabeled phosphorylated peptide, the polarization diminished to less than 10% of control. The competition of labeled tracer and the unlabeled peptide as a function of the concentration of the unlabeled peptide is further shown in Figures 8 and 9. In Figure 8, 1 nM tracer and 0.3 µg/mL anti-pY antibody are incubated in 150 mM NaCl, 50 mM Hepes, pH 7.6, and 0.01% bovine IgG in the presence of various amounts of unlabeled phosphorylated peptide. As shown, over the range of 0-700 nM, the unlabeled peptide competes as a function of concentration with the tracer. (Here, "tracer" refers to the phosphorylated peptide set forth above carrying the luminescent

label, and "unlabeled peptide" refers to the same peptide without the fluorescein conjugate.)

Figure 9 shows the competition curve in the presence of 75 nM tracer and 5 µg/mL anti-pY antibody as a function of unlabeled peptide concentration.

- 5 This competition curve is indicative of the concentration dependence on unlabeled competitor over the range of 0-1 µM.

Example 2

Measurement of Kinase Activity

- Several peptides were used as substrates for phosphorylation. These
10 peptides were:

KS2: biotin-EGPWLEEEEEAYGWMDF-amide (Boehringer-Manheim Catalog No. 1768719);

D-peptide: DYMTMQIG;

S-peptide: SRGDYMTMQIG.

- 15 Each of these substrates at about 10 µM was incubated with or without 200 ng of insulin receptor cytoplasmic kinase domain (IR-CKD) in 100 µL assay buffer (15 mM Tris, pH 7.5, 0.05% BSA, 2 mM MnCl₂, 10 mM MgCl₂, 200 µM ATP) for one hour and then stopped by bringing the solution to 20 mM EDTA. This is the "catalysis mixture."

- 20 Aliquots ranging from 10-50 µL of the catalysis mixture were added to tubes containing 0.8 mL luminescence polarization (FP) buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.01% bovine IgG, 1 mM sodium vanadate, 20 mM EDTA) containing 1.0 nM of the tracer of Example 1. A 0.3 µg portion of anti-pY (4G10) was added to each tube. The tubes were vortexed and incubated for
25 15 minutes in the dark at room temperature. Using a standard calibration curve, the amount of modified substrate was determined. As shown in Table 1, KS2 was a better substrate than either of the D or S peptide essentially by a factor of 10.

Table 1 nMol pY Created in Original Reaction Volume				
Tube No.	S-peptide	D-peptide	KS2-peptide	KS2 but no CKD
1	569	639	5411	66
2	727	625	5772	74
3		593	6412	
4			6046	
5			6452	
PmoL pY/min/ μ g CKD	4.9	4.7	46.7	<0.7

The results obtained in the foregoing assay were evaluated when various amounts of the catalysis mixture were added to the tubes for the luminescence polarization competition reaction. The results are shown in Figure 10, which is mainly a showing of the signal as proportional to the concentration of CKD. As expected, if the reaction mixture did not contain CKD or did not contain substrate, no reduction of polarization was seen. However, if both CKD and substrate were present, the level of reduction of polarization decreased with the amount of catalysis mixture added.

To measure kinase activity quantitatively, rather than simply the necessity for kinase and its substrate, the foregoing experiments were repeated but sampled at various times and using various CKD concentrations. The results are shown in Figure 11, which is mainly a showing of the signal at specific concentrations of CKD as proportional to time. As shown, higher concentrations of CKD enhanced the rate of diminution of luminescence polarization in the reaction mixture.

Example 3

Effect of Agonists and Antagonists

The foregoing experiment, in which luminescence polarization was measured as a function of time, was repeated with CKD at 4 μ g/mL concentration in the presence of a known inhibitor, piceatannol, as measured at a fixed time using 5 μ L of the catalysis mixture. The results are shown in

Figure 12, indicating that the rate of diminution of luminescence polarization is diminished in the presence of the inhibitor.

The opposite result in the presence of a known agonist of the insulin receptor is shown in Figure 13. As indicated, higher concentrations of TER
5 16998, known to stimulate the insulin receptor, at increasing concentrations provided significant decreases in maximal polarization when this determination was run as an end-point assay using 5 μ L of the catalysis mixture.

A similar assay can be used to determine kinase activity of alternative receptors, such as the IGF1r receptor as shown in Figure 14. The results in this
10 figure show that the decrease in maximal polarization can be used as a measure of either IR-CKD activity or IGF1r-CKD activity.

Example 4

Peptide Mapping

Phosphorylated IR-CKD (83 μ g) was hydrolyzed with endoproteinase
15 Lys-C lysyl endopeptidase protease (EC. 3.4.21.50), (Wako Chemical USA, Inc., Richmond, VA), and the reaction mixture subjected to High Pressure Liquid Chromatography (HPLC) using a Phenomenex (Torrance, CA) Jupiter 5 micron C18 300 \AA 250 \times 2.0 mm reversed phase column, with detection at 214 nm. The samples were eluted using a continuous elution gradient beginning
20 with 95% Buffer A-5% Buffer B to 5% Buffer A-95% Buffer B. The composition of Buffer A was: 5% acetonitrile; 0.1% trifluoroacetic acid (TFA); 95% water. The composition of Buffer B was: 95% acetonitrile; 0.085% trifluoroacetic acid (TFA); 5% water.

The fractions representing individual peptide peaks seen in Figure 15
25 were assayed as described in Example 3 for the presence of phosphorylated tyrosine in the fragment. As shown in Figure 16, fractions 6, 7, 12, 13, 18, 21, 22, and 23 contain phosphorylated peptides.

Example 5Serine/threonine (S/T) Kinase Assays

The invention provides apparatus, methods, compositions, and kits for determining the presence and activity of S/T kinases. The methods may include
5 homogeneous luminescence assays, so that difficulties and complexities associated with heterogeneous assays and/or radioactivity may be avoided. The methods, compositions, and kits may include one or more generic kinase substrates, and binding partners that interact with that substrate and/or phosphorylated forms of that substrate. The invention is particularly well suited
10 to screening for modulators of S/T kinase activity, as in drug discovery applications, although the invention also may be used for other applications.

Methods provided by the invention generally include the following steps:

- 15 (1) providing a protein substrate having one or more sites capable of being phosphorylated by an S/T kinase;
- (2) providing a phosphate donor capable of donating a phosphate group to the protein substrate in the presence of
20 an S/T kinase;
- (3) providing a specific binding partner capable of specifically binding the phosphorylated protein substrate, but generally incapable of specifically binding the unphosphorylated
25 protein substrate, or vice versa;
- (4) contacting a sample with the protein substrate, phosphate donor, and specific binding partner, so that the protein substrate is phosphorylated if the sample includes S/T
30 kinase activity; and

- (5) detecting S/T kinase activity by detecting changes in interactions between the protein substrate and the specific binding partner.

5 The protein substrate generally comprises any amino acid, peptide, or protein, or fragment, derivative, or analog thereof, capable of being phosphorylated by an S/T kinase. Such amino acids, peptides, proteins, or fragments, derivatives, or analogs thereof, preferentially will include one or more serine and/or threonine residues.

10 The protein substrate may include a generic substrate capable of being phosphorylated by a variety of S/T kinases. Preferred generic substrates may include histone type III-ss, human/bovine myelin basic protein (MBP), and fragments of MBP including all or part of amino acids 4-14. The lattermost substrate has the following sequence:

15 EKRPSQRSKYL

Here (and throughout the application), S is the one-letter abbreviation for serine, T is the one-letter abbreviation for threonine, and E, K, R, . . . are the
20 one-letter abbreviations for other amino acids, as specified in the Appendix. Here, S and T are underlined so that the location(s) of potential phosphorylation sites may be more easily identified. Preferred substrates also may include serine and/or threonine amino acids followed in the C-terminal direction by a proline residue, particularly immediately after (i.e., within one
25 amino acid) of the serine or threonine. Preferred substrates also may include amino acid sequences having one or more serine and/or threonine amino acids, enriched for several amino acids to one or both sides of at least one serine and/or threonine by basic amino acids, such as lysine, arginine, and histidine, particularly within about three amino acids in the C-terminal direction. Basic
30 amino acids, including lysine, arginine, and histidine, generally are positively

charged under physiological conditions. These substrates are preferred because they may have enhanced recognition by kinase enzymes.

Preferred substrates generally are short, so that they may undergo a more significant change in size and hence rotational correlation time upon
5 binding to a binding partner (such as an antibody). Here, short generally means shorter than about 40 amino acids, and especially shorter than about 15 amino acids.

The phosphate donor generally comprises any group capable of donating a phosphate group to the protein substrate in the presence of an S/T kinase. A
10 preferred phosphate donor is adenosine triphosphate, or ATP.

The specific binding partner generally comprises any compound capable of specifically binding the phosphorylated protein substrate, but incapable of specifically binding the unphosphorylated protein substrate, or vice versa. Specific binding means binding to the specific binding partner to the exclusion
15 of binding to most other moieties. Specific binding can be characterized by a binding coefficient. Generally, specific binding coefficients range from 10^{-4} M to 10^{-12} M and lower, and preferred specific binding coefficients range from 10^{-9} M to 10^{-12} M and lower.

In some assays, preferred specific binding partners, or fragments, derivatives, or analogs thereof, may be coupled to solid supports or other
20 moieties, including beads and walls. In other assays, fragments, derivatives, or analogs of preferred specific binding partners may be used, if such fragments, derivatives, and analogs retain their specificity and binding affinity for their binding partners.

25 Preferred specific binding partners include immunological specific binding partners, such as antibodies. In particular, preferred specific binding partners include antibodies against unphosphorylated or phosphorylated forms of the preferred protein substrates listed above, including antibodies against the following phosphorylated MBP fragments:

EKRPpSRSKYLEKRPSQRpSYLEKRPpSRpSYL

- 5 Here, a "p" is used to denote phosphorylation of the subsequent amino acid. Preferred specific binding partners also include antibodies against the following phosphorylated peptides:

KRREILSRRpSYRKFpTLPLQKHFPQFpSSSRKRpTLRRLpSPELERLIQCLRRApSLGGSPSVRCSpSMpSKKLNRTLpSVASLRSRHSpSYPAGTRPRAApTF-NH₂LpTPLKLRRApSLG-NH₂

- 10 The lattermost two peptides are amidated at the C-terminal, which may act to stabilize the peptides.

- These or other protein substrates, or fragments, analogs, and/or derivatives of such substrates, or cells expressing such substrates, fragments, analogs, and/or derivatives can be used as immunogens to produce
- 15 immunological binding partners thereto. Generally, preferred immunogens are phosphorylated forms of preferred substrates, especially substrates in which phosphorylated serines and/or threonines are flanked by basic amino acids, because serine, threonine, and phosphate groups alone may be too generic to induce good immune responses. Immunological binding partners include
- 20 polyclonal and monoclonal antibodies. Immunological binding partners also include chimeric, single chain, and humanized antibodies, as well as Fab fragments and the products of Fab expression libraries.

Immunological binding partners, including antibodies and fragments, analogs, and/or derivatives of such antibodies, can be produced by various

generally known procedures. For example, antibodies against protein substrates can be obtained by injecting or otherwise administering the protein substrates into an animal, where the animal preferably is different in species than the animal from which the protein substrate is derived. The antibody so obtained
5 will then bind the protein substrates. In this manner, even a sequence encoding only a fragment of a protein substrate can be used to generate antibodies that will bind the whole protein substrate.

Monoclonal antibodies can be prepared by any technique that provides antibodies produced by continuous cell line cultures. Examples include the
10 hybridoma technique (Köhler and Milstein, *Nature* 256:495 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985)).

15 Single-chain antibodies can be prepared using any technique for producing single-chain antibodies. Examples include the method described in U.S. Patent No. 4,946,778.

The sample generally comprises any composition for which the presence or activity of S/T kinases is to be tested. The sample may be natural, artificial,
20 or a combination thereof. The sample may include proven or putative S/T kinases, including cyclic AMP-dependent kinase (A-kinase), cyclic GMP-dependent kinase (G-kinase), protein kinase C (C-kinase), Ca^{2+} -calmodulin-dependent kinase (CaM-kinase), phosphorylase kinase, MAP-kinase, and TGF- β receptor, among others. The sample also may include proven or putative
25 modulators of S/T kinases, including inhibitors and/or enhancers of S/T kinases.

The sample may be contacted with the protein substrate, phosphate donor, and specific binding partner using any method for effectuating such contact. A preferred method is by mixing the materials in solution, although

other methods such as attaching one or more components to a bead or surface also may be used.

S/T kinase activity within the sample may be detected using any method for detecting specific binding between the unphosphorylated or phosphorylated
5 protein substrate and the specific binding partner. A preferred method is a luminescence assay, especially a homogeneous luminescence polarization assay.

Luminescence S/T kinase assays are constructed so that interaction of the protein substrate and specific binding partner may be correlated with some
10 change in luminescence. In particular, luminescence assays are constructed to detect changes in the interaction between the protein substrate and specific binding partner brought about by phosphorylation of the protein substrate by S/T kinases, and to qualitatively or quantitatively correlate such changes with such activity. Suitable assays may measure intrinsic or extrinsic properties of
15 the luminescence, and may include noncompetitive, competitive, and sandwich assays.

Luminescence assays may be performed by collecting luminescence light from luminophores associated with the protein substrate, specific binding partner, and/or other moieties whose luminescence reflects interactions of the
20 protein substrate and specific binding partner. Such luminophores may be intrinsic to the protein substrate, specific binding partner, and/or other moieties, such as tryptophan, tyrosine, or luminescent gene products such as green fluorescent protein. Such luminophores also may be extrinsic, as described below. Extrinsic luminophores may be associated with the protein substrate,
25 specific binding partner, and/or other moieties covalently or noncovalently.

The compositions and kits provided by the invention include but are not limited to compositions and kits for practicing the methods described above. For example, compositions and kits may include protein substrates, specific binding partners, and fragments, analogs, and derivatives thereof. Compositions and kits

also may include reactive and luminescently labeled versions of such protein substrates, specific binding partners, fragments, analogs, and derivatives.

The methods, compositions, and kits provided by the invention may be practiced using apparatus, methods, and compositions described in the above-identified patent applications, which are incorporated herein by reference. For example, luminescence may be detected using high-sensitivity luminescence apparatus, including those described in U.S. Patent Application Serial No. 09/062,472, filed April 17, 1998, U.S. Patent Application Serial No. 09/160,533, filed September 24, 1998, and PCT Patent Application Serial No. PCT/US98/23095, filed October 30, 1998. Luminescence also may be detected using high-sensitivity luminescence methods, including those described in PCT Patent Application Serial No. PCT/US99/01656, filed January 25, 1999, and PCT Application Serial No. PCT/US99/03678, filed February 19, 1999. Luminescence also may be detected using sample holders optimized for performance with the above-identified high-sensitivity luminescence apparatus and methods, including those described in PCT Patent Application Serial No. PCT/US99/08410, filed April 16, 1999.

Further disclosure relating to S/T kinase assays is given in U.S. Provisional Patent Application No. 60/138,438, which is incorporated herein by reference.

Example 6

Tyrosine Kinase Assays

TK enzymes function by phosphorylating tyrosine residues on substrate proteins or peptides. The amount of phosphorylated protein in a solution containing TK enzymes thus may be used as an assay of TK enzyme activity. TK activity may be quantified by a competitive assay. In a competitive TK assay, as shown schematically in Figure 17, proteins phosphorylated by TK enzymes ("enzyme product") compete with a luminescently labeled phosphotyrosine analog ("tracer") for binding sites on an anti-phosphotyrosine

antibody ("antibody"). The tracer has low luminescence polarization when free and high luminescence polarization when bound to antibody. Hence, luminescence polarization will be low when TK enzyme activity is high, because enzyme product will be present to compete tracer off the antibody.

5 Conversely, luminescence polarization will be high when TK enzyme activity is low, because enzyme product will not be present to compete tracer off the antibody.

A known TK assay includes six steps, as follows:

- 10 1) Run kinase reaction in a microplate
- 2) Stop kinase reaction with chelating EDTA solution
- 3) Pipette an aliquot of the stopped reaction mixture into a second microplate
- 4) Add antibody to second microplate
- 15 5) Add tracer to second microplate
- 6) Measure luminescence polarization in second microplate

Although this six-step assay is adequate to measure TK enzyme activity, it has a number of shortcomings. The assay involves a relatively large number of

20 steps, increasing the likelihood of error. Moreover, the assay requires that sample be transferred between different containers, increasing waste and cost and further increasing the likelihood of error. These shortcomings are particularly significant if the assay is used to screen new drugs, because such screening typically involves conducting massive numbers of assays, frequently

25 hundreds of thousands. Under such conditions, throughput is significantly decreased by having to conduct six steps for each assay, and waste is significantly increased by having to use two sample containers for each assay.

The invention may address these and other shortcomings by providing assays for TK enzyme activity that require fewer than six steps and that involve

30 only a single sample container.

In a first embodiment, the invention includes a TK assay involving only five steps and only a single container. This assay includes the following steps:

- 1) Run kinase reaction
- 2) Add a stop solution to stop kinase reaction
- 3) Add antibody
- 4) Add tracer
- 5) Measure luminescence polarization

In a second embodiment, the invention includes a TK assay involving only four steps and only a single container. This assay includes the following steps:

- 1) Run kinase reaction
- 2) Add a stop solution to stop kinase reaction
- 3) Add a mixture of antibody and tracer
- 4) Measure luminescence polarization

Figure 18 shows results generated with the second protocol. Competitive curves demonstrate assay performance in which a mixture of tracer and antibody is added to the simulated enzyme product (o-phospho-L-tyrosine). The tracer and antibody were incubated together in the mixture for 30 minutes to equilibrate before addition to the phosphotyrosine in the assay plate. Plates 2, 3, 5, and 6 correspond to this method, with the same final antibody/tracer solution prepared by different intermediate dilutions. Plates 1 and 4 were run according to the first simplification. Plates 1-3 and 4-6 were run on two separate days.

In a third embodiment, the invention includes a TK assay involving only three steps and only a single container. This assay includes the following steps:

- 1) Run kinase reaction
- 2) Add a mixture of stop solution to stop kinase reaction, antibody, and tracer
- 3) Measure luminescence polarization

Figure 19 shows the results generated with the third protocol. Competitive curves demonstrate assay performance in which a mixture of EDTA, tracer, and antibody is added to the simulated enzyme product (o-phospho-L-tyrosine).

The EDTA, tracer, and antibody were incubated together in the mixture for 30 minutes to equilibrate before addition to the phosphotyrosine in the assay plate. In plates 2 and 3 the concentration of EDTA was 20 mM; in plates 4 and 5 it was 50 mM. In plates 2 and 4, the phosphotyrosine solution to which the mixture was added had no divalent cations (necessary for kinase activity). In plates 3 and 5 the phosphotyrosine plates did contain divalent cations, more fully simulating actual assay conditions. Plate 1 was a control, run according to the first simplification.

These methods should reduce significantly the time, waste, and cost associated with conducting TK assays, with no significant loss in biochemical performance. In each embodiment, the step to run the kinase reaction may include running the reaction in a microplate. In addition, the step to add a stop solution may include adding a stop solution containing a chelator, such as EDTA.

These TK assays, particularly the second and third, yield surprisingly good results. The reason may depend in part on the relative time scales of the assay and on the kinetics of the equilibration of the tracer-antibody and product-antibody interactions. If the time scale of the equilibration is the slower, and if pre-formed tracer-antibody complex is added to product, the product may not fully displace the tracer before the luminescence-polarization is measured. If the antibody is added to the enzyme product, and then the tracer is added, equilibration may still be incomplete, but at least the enzyme product may block binding of tracer in a fashion directly related to its concentration. If the tracer and enzyme product are incubated together and then brought into contact with the antibody, equilibrium should be reached as long as the rate constants for antibody binding are similar for the tracer and enzyme product.

If the equilibration rate is relatively rapid, the order in which enzyme product, tracer, and antibody come into contact should be immaterial: the same

equilibrium should be reached in all cases. It is, however, well known that antibody/antigen equilibration times can be long.

Figures 20-21 show results generated in additional studies of tyrosine kinase activity, showing how luminescence polarization may be used in a homogenous immunoassay format to screen for a modulator (inhibitor) of enzyme activity. Specifically, anti-phosphotyrosine antibody (1:750 dilution of ascites fluid) and fluorescein-labeled phosphotyramine (1 nM) were incubated with the indicated concentrations of phosphorylated tyrosine-kinase substrate (i.e., the enzyme product). The enzyme product competitively released the labeled compound from the antibody, decreasing the luminescence polarization, because the polarization of the label is high when bound to the antibody and low when displaced from the antibody by the enzyme product. Thus, the higher the enzyme activity, the lower the luminescence polarization.

Figures 20-21 show results obtained under a variety of assay conditions. Figure 20 shows results for 200 μ L samples in 96-well microplates and 5 μ L samples in 1536-well microplates. Error bars, averaging 4 to 8 mP, are standard deviations. The phosphotyrosine IC_{50} values determined from the data are 379 ± 22 nM for the 200 μ L samples and 326 ± 30 nM for the 5 μ L samples. Error bars are standard errors of the mean. Figure 21 shows results for 40, 60, 80, and 100 μ L samples in 384-well microplates. Error bars, averaging 1 to 4 mP, are standard deviations. Error bars for polarizations in Figures 20-21 should be compared with the maximum change in polarization for the assay, which is about 110 mP.

Further disclosure relating to tyrosine kinase assays is given in U.S. Provisional Patent Application No. 60/092,203, which is incorporated herein by reference.

Example 7

Phosphatase Assays

The invention also may be used to detect phosphatases rather than kinases if the protein substrate initially is phosphorylated and if the specific
5 binding partner interacts with the substrate to detect dephosphorylation, for example, as shown in Figure 5.

Example 8

Peptide Tracers for Tyrosine Kinase Assays

In still another aspect, the invention is directed to specifically designed
10 peptides that show enhanced affinity for binding to the 4G10 antibody, which is directed to phosphorylated tyrosine. Phosphorylated tyrosine is an indicator of an activated form of the insulin receptor and many other receptors such as EGF-receptor, and IGF-1 receptor, as well as nonreceptor tyrosine kinases such as src, fyn and many others known in the art. These peptides are of the formula

15

FL-A-pY-TGLSTRNQET-pY-ATH-NH₂

FL-pY-pY-pY-IE-NH₂

FL-G-pY-NELNLGRREE-pY-DVL-NH₂

20 Here, Y is the one-letter abbreviation for tyrosine, pY is phosphorylated tyrosine, and A, T, G . . . are one-letter abbreviations for other amino acids, as specified in the Appendix.

Preferred embodiments for FL include various FL-S moieties, especially the 5-carboxy and 6-carboxy derivatives of fluorescein, which readily form
25 amides at the N-terminus of the peptide.

These peptides are suitable tracers for competition assays with respect to analytes that contain phosphorylated tyrosine, such as the activated insulin receptor and natural or artificial substrates of it. They are advantageous over peptide labels known in the prior art by virtue of their enhanced affinity for
30 specific binding partners for phosphorylated peptides containing

phosphotyrosines. The assay methods may be extended to phosphoserine/threonine as well.

The peptides are thus useful as tracers in any luminescence polarization assay for phosphorylated peptides wherein the phosphorylation is associated with tyrosine residues. The peptides are added to a sample that contains or is suspected to contain such phosphorylated peptides along with an opposite member of a specific binding pair involving these peptides. The peptides compete with any phosphorylated protein in the sample for the opposite member so that the degree of luminescence polarization observed in light emitted from the sample is inversely correlated with the concentration of phosphorylated protein in the sample. This determination can also be made as a function of time if the activity of an enzyme that phosphorylates proteins is to be measured. In addition, the effect of compounds on such phosphorylation reactions can be studied by conducting the assay in the presence and in the absence of candidate compounds.

Example 9

Metal-Based Assays

The presence and activity of kinases and phosphatases can be determined using assays capable of detecting phosphorylated and dephosphorylated protein, as described above. These assays may involve monitoring changes (e.g., increases or decreases) in the interactions between a protein substrate and a binding partner capable of recognizing the phosphorylated substrate but not the dephosphorylated substrate, or vice versa. In particular, these assays may employ specific binding partners such as antibodies that recognize only particular phosphorylated or dephosphorylated substrates (such as a phosphorylated tyrosine, serine, or threonine surrounded by a particular sequence of amino acids). Thus, these assays typically require careful matching of the substrate and binding partner, potentially limiting the substrates to those for which a specific binding partner is available.

The assays described here may circumvent these limitations by permitting the use of relatively nonspecific binding partners in phosphorylation assays. Such binding partners may recognize phosphorylated protein substantially without regard to the particular phosphorylated amino acid or surrounding amino acid sequence. Examples of such binding partners include metal cations, and especially trivalent metal cations such as Fe^{3+} (iron) and Ga^{3+} (gallium). Examples also may include such binding partners complexed with other compositions, including macromolecules and/or surfaces. Potential difficulties with this approach include (1) interference from compounds (e.g., ATP, free phosphate, EDTA, and possibly primary/secondary amines) that may compete with or otherwise affect the interaction between the metal and the phosphorylated protein, and (2) difficulty in maintaining a pH that preserves the affinity and selectivity of the binding between the metal and phosphorylated protein.

Changes in the interactions between the protein substrate and (specific or nonspecific) binding partner brought about by enzyme activity may be monitored using any suitable assay format and technique. For example, changes may be monitored using competition or sandwich assays, and homogeneous or heterogeneous assays, including heterogeneous assays using plates or beads. Changes also may be monitored using luminescence-based techniques, including energy-transfer and polarization-based techniques. Changes also may be monitored using microfluidics-based techniques.

Further aspects of the invention are illustrated without limitation in the following examples:

Example 9A

Ru^{2+} was entrapped in a small (~20-30 kDa) synthetic polymer macromolecule (MM) obtained from Presens (Germany). The macromolecule is relatively hydrophilic, with carboxyl groups on its surface for activation. The MM with the entrapped Ru^{2+} was used as a support to immobilize trivalent

metal cations, including Fe^{3+} and Ga^{3+} . A kinase enzyme phosphorylated a luminescently labeled kinase substrate, which bound to the metal cations immobilized on the MM. Binding was detected using polarization and energy transfer techniques. Specifically, binding led to a decrease in substrate mobility and a concomitant increase in the polarization of light emitted by the luminophores bound to the substrate. Similarly, binding led to a decrease in separation between the luminophores bound to the substrate and the Ru^{2+} immobilized in the MM, and a concomitant increase in energy transfer from the Ru^{2+} (donor) to the luminophore (acceptor). Polarization and energy transfer can be detected using apparatus and methods described in patent applications listed above under Cross-References, which are incorporated herein by reference.

This approach may be extended through various changes and modifications. For example, in polarization assays, the Ru^{2+} is irrelevant and may be omitted, because the MM is just a carrier. In energy transfer assays, the approach may be extended to any energy transfer pair, including a lanthanide. Also, the luminescent donor need not be encapsulated in the MM. One could attach a luminescent species directly to a suitable Fe^{3+} or Ga^{3+} chelate. In a heterogeneous assay, phosphorylated proteins bound via Ga^{3+} or Fe^{3+} binding as above to microplates, particles, or inner surfaces of microfluidic devices may be detected after a wash by measuring luminescence intensity. Such detection can take place either directly on the surfaces or in the solution phase by adding an elution solution such as a phosphate buffer. With other detection methods, such as the laser-scanning method used in FMATTM technology by PE Biosystems, the bound phosphoproteins can be detected directly on the beads, without the need for washing or separation. Other labels such as enzymes also may be used in the heterogeneous format.

Example 9B

The chelator imidodiacetic (IDA) acid was linked to the MM using the secondary amine group of IDA and a carboxyl group on the MM. Afterwards, the MM-IDA was incubated with either FeCl_3 or GaCl_3 . It was found that the FeCl_3 quenches the luminescence of Ru, while the GaCl_3 does not.

The macromolecule loaded with Ga^{3+} (MM-Ga) was tested for the binding of the following di-(phosphotyrosine) peptide fluorescein-labeled tracer, denoted tyrosine kinase 1 (TK-1) tracer: FL-A-pY-TGLSTRNQET-pY-ATH-NH₂.

Figure 22 shows the effects of incubating 10 nM TK-1 tracer with different concentrations of MM-GA (total volume = 50 μL ; incubation time = 60 minutes). The maximum polarization change was more than 200 mP when the MM-Ga and TK-1 tracer were incubated in MES buffer (0.1M MES, pH 5.5, 1.0 M NaCl).

Figure 23 shows a dose-response curve for ATP, with 10 nM TK-1 tracer and 1.6 nM (estimated) MM-Ga. The bound/total ratio of the tracer was calculated based on the measured luminescence polarization value. Here, the IC₅₀ for ATP was around 10 μM , in an assay with 10 nM of tracer and 1:4000 dilution of MM-Ga (final concentration).

Figure 24 shows a dose-response curve for TK-1 calibrator, with 10 nM TK-1 tracer and 1.6 nM (estimated) MM-Ga. The TK-1 calibrator is the same phosphopeptide as TK-1 tracer without a fluorescein label. The bound/total ratio was calculated as in Figure 23. The IC₅₀ for the TK-1 calibrator also was around 10 μM .

The macromolecule loaded with Ga^{3+} (MM-Ga) also was tested for the binding of the following fluorescein-labeled monophosphoserine peptide tracer, denoted serine/threonine kinase 1 (STK-1) tracer: FL-RFARKG-pS-LRQKNV.

Figure 25 shows binding of STK-1 tracer to various concentrations of MM-Ga (STK-1 tracer concentration = 5 nM; total volume = 50 μ L; incubation time = 70 minutes).

Figure 26 shows the effects of a HEPES-based buffer on the MM-Ga/phosphopeptide interaction (STK-1 tracer concentration = 5 nM; MM-Ga is diluted 1:1000). The buffer included 140 mM NaCl, 20 mM HEPES, and 0.1% bovine gamma globulin. The results show that such buffers interfere with the MM-Ga/tracer interaction.

Example 9C

We demonstrated the specificity of the binding between phosphorylated peptide and a macromolecule activated with Ga (III) metal ion (MM-Ga) and its potential in the development of generic kinase assays. The materials included cAMP-dependent protein kinase A (PKA, from Promega) as the enzyme and fluorescein-labeled Kemptide (fluo-Leu-Arg-Arg-Ala-Ser-Leu-Gly) as the substrate.

(1) Endpoint assay. Figure 27 shows an endpoint assay of PKA activity with MM-Ga under the following conditions: (1) reaction with enzyme, assay with MM-Ga; (2) reaction with enzyme, no MM-Ga; (3) no enzyme control, with MM-Ga; and (4) no enzyme control, no MM-Ga. The assay was performed as follows. First, a mixture was prepared of 20 mM $MgCl_2$, 0.2 mM ATP, 2 mM $NaVO_4$, and 100 μ M fluo-Kemptide in a total of 50 μ L 40 mM Tris-HCl, pH 7.4. Second, the reaction was initiated by adding 1.0 μ L of the enzyme PKA to the mixture; for a control reaction, no PKA was added. Third, the reaction was run overnight at room temperature. Fourth, the reaction and control were diluted 1:1000, and 1 μ L of the diluted solution was added to a volume of 49 μ L of MM-Ga solution (approx. 30 nM MM-

Ga) in a MES buffer (pH 5.5) in a 384-well plate. Fifth, the plate was incubated at room temperature for 60 min. Finally, the luminescence polarization was measured using an LJL Analyst™ light-detection platform.

5

(2) Time-course assay. Figure 28 shows a time-course assay of PKA activity with MM-Ga performed under the reaction conditions of Figure 27. At preselected times, 1 μL of reaction mixture was taken out from the reaction and immediately diluted into a volume 1000 μL of MES buffer. Afterwards, 1 μL of each diluted sample was added to a volume of 49 μL of MM-Ga solution, and an assay was conducted as described above.

10

The results show that phosphorylated peptide binds specifically to MM-Ga. Figures 27 and 28 show that MM-Ga does not bind to non-phosphorylated Kemptide but binds strongly to phosphorylated Kemptide. Figure 28 shows the potential of using the MM-Ga in a kinase reaction.

15

Example 9D

The metal ions (e.g., Ga^{3+}) immobilized on the macromolecules in the PKA/Kemptide system can bind selectively to phosphorylated peptide generated in a kinase reaction. Using the IMAP (immobilized metal with affinity to phosphoproteins) system, we were able to monitor the time course of a kinase reaction using a homogeneous luminescence polarization (FP) assay. In particular, the polarization changed from 50 mP before the reaction was started to 300 mP after the reaction was completed.

25

Alternatively, the IMAP technology can be used in a heterogeneous assay format with a metal-coated plate. Here, the feasibility of using metal-coated plates in the development of generic kinase assays is demonstrated with a commercial Ni^{2+} -coated plate, in which the Ni^{2+} has been replaced with Ga^{3+} . 200 μL 0.5 M EDTA-containing solution was added to each well of a 96-well

30

Ni²⁺-coated plate, and the plate was incubated at room temperature for 1 hour. The process was repeated two more times to remove all of the Ni²⁺ from the plate. The plate was then washed 3 times with 10 mM Tris buffer, pH 7.4. Next, 200 μ L 0.1 M GaCl₃ solution was added to the each well of the plate, and
5 the plate was incubated at room temperature for overnight. The plate was washed three times before being used in a kinase assay.

In the assay, a kinase reaction was set up as previously described, using PKA as the enzyme and fluorescein-Kemptide as the substrate. At each time point, 1 μ L was taken from the reaction and diluted into 1000 μ L of MES
10 buffer. Later, a volume of 100 μ L of each diluted solution was added to the Ga³⁺-coated plate, and incubated for 1 hour at room temperature. The plate was then washed three times, and 100 μ L of a 1 M KH₂PO₄ solution was added to elute the bound phosphorylated Kemptide from the plate. The fluorescence intensity was measured with an LJL Analyst™ light-detection platform set in
15 fluorescence intensity mode and using a medium attenuator. In a control assay, an unmodified Ni²⁺-coated plate was used. The results are shown in Figure 29.

Although this is a heterogeneous assay format, which requires several washing steps, it offers the advantages of a generic assay that is applicable in principle to any kinase regardless of its substrate specificity. This may save
20 assay developers 3 to 6 months of time and effort in making antibodies that recognize specifically a phosphorylated version of an amino acid sequence. The lack of availability of such special antibodies often is the major obstacle in the development of non-radioactive kinase assays.

Thus, for high-throughput screening applications, there are several
25 possible assay designs that take advantage of MM-Ga in the development of generic kinase assays. These assays do not rely on a specific anti-phosphoprotein antibody and are applicable to a wide variety of kinases regardless of their substrate specificity.

Example 10

Tyrosine-Kinase Assay With BODIPY-TMR Tracer

Figure 30 shows polarization calibration curves for TK-1 with BODIPY-TMR tracer, for (A) 500 msec and (B) 200 msec integration times.

5 Experimental conditions were as follows:

Tracer = 0.4 nM TK-1 peptide, labeled with TMR-Bodipy (final)

Ab = 1:400 (1:1600 final)

1 hour incubation; 500 msec integration

Measured values of IC₅₀ were 33 nM for (A) and 32 nM for (B). Results are essentially the same as those obtained with a fluorescein label.

10

Example 11

Integrated Cell-signaling Assays

This example shows assays for integrated cell signaling mechanisms. Specifically, the assays described here for kinase and phosphatase enzymes may be performed in combination with the assays for cyclic nucleotides and GTP-binding proteins described in PCT Patent Application Serial No. ___, filed
15 June 9, 2000, entitled *Cell-Signaling Assays*, of inventors J. Richard Sportsman and Lawrence M. Kauvar, which is incorporated herein by reference. Such combination assays permit study of signaling mechanisms involving multiple pathways.

20

Example 12

Improved-signal Assays

This example shows assays with improved signals, signal-to-noise ratios, and/or signal-to-background ratios.

Signal may be enhanced in several ways, including (1) using a high
25 color temperature light source, such as a xenon arc lamp, in a continuous illumination mode, (2) using a dichroic or multi-dichroic beamsplitter, and/or (3) using a sample holder whose shape is "matched" to the shape of the optical

beam of the instrument, especially if the sample holder is elevated to bring the sample closer to a detector. The high color temperature light source increases the number of usable photons, which is important because the lower limit of the signal-to-noise ratio is set by the square root of the total number of photons collected in the measurement. These enhancements are described in more detail in the following U.S. Patent Applications, which are incorporated herein by reference: Serial No. 09/349,733, Serial No. 09/478,819, and Serial No. 09/494,407.

Signal-to-noise ratios can be enhanced at least in part by increasing signals, for example, by using the techniques described in the previous paragraph.

Signal-to-background ratios can be enhanced in several ways, including (1) using confocal optical systems having a sensed volume to avoid luminescence from the microplate walls, (2) selecting a microplate or other substrate that increases the signal and reduces the luminescent background from materials in the microplate, (3) selecting the light sources, luminescence filters, optics, signal collection electronics, and mechanical system used in the luminescence detection optical system for maximum signal-to-background ratio, and (4) utilizing signal processing, background subtraction, and luminescence lifetime techniques, particularly FLAMe™ methodology for background reduction, as described below. These enhancements are described in more detail in the following U.S. Patent and U.S. Patent Applications, which are incorporated herein by reference: Patent No. 6,071,748, Serial No. 09/349,733, Serial No. 09/478,819, and Serial No. 09/494,407.

Example 13

Improved-polarization Assays

This example shows mechanisms for increasing the change in polarization that accompanies a change in binding, so that the change in binding can be measured more easily. These mechanisms may be used in any

of the assays described here involving luminescently labeled species, such as labeled peptide tracers, among others.

The change in polarization upon binding can be increased by making any linker between the luminophore and the labeled species (e.g., the peptide tracers) as short and/or rigid as possible, while maintaining relevant substrate properties for the enzymes involved in the assay. Short and/or rigid linkers will restrict luminophore motion relative to the labeled species, reducing the “propeller effect” so that the luminophore more accurately reports the motion of both the free and bound labeled species. The rigidity of the linker may be increased by avoiding using hexanoic acid linkers, which typically are long and flexible, and by using amide groups in place of methylene groups, among other mechanisms.

The change in polarization upon binding also can be increased by including an appropriately positioned energy transfer acceptor on the binding partner, so that energy transfer will occur from the luminophore to the acceptor upon incorporation. Such energy transfer will shorten the lifetime of the luminophore, thereby increasing its polarization (because polarization varies inversely with lifetime, all else being equal).

The change in polarization upon binding also can be increased by decreasing the mobility of the binding partner for the labeled species. Mobility can be decreased by increasing the size of the binding partner, either directly or by forming a complex with a mass label. Suitable mass labels include other molecules and beads, among others. The use of mass labels is described in detail in PCT Patent Application Serial No. PCT/US99/24707, which is incorporated herein by reference. Mobility also can be decreased by attaching the binding partner to a surface, such as the surface of a sample holder. Attachment to other molecules, beads, and/or surfaces may be accomplished using any of a number of well-known reactive groups.

Example 14

Principles of Luminescence Polarization Assays

This example describes principles of luminescence polarization assays. Here, luminescence refers to the absorption and subsequent re-emission of light by a luminescent molecule, or "luminophore," and polarization refers to the direction of the light's electric field, which generally is perpendicular to the direction of the light's propagation. Luminescence includes fluorescence and phosphorescence, among others. In a luminescence polarization assay, specific molecules within a composition are labeled with one or more luminophores.

5 The composition then is illuminated with polarized excitation light, which preferentially excites luminophores having absorption dipoles aligned parallel to the polarization of the excitation light. These molecules subsequently decay by preferentially emitting light polarized parallel to their emission dipoles. The extent of polarization of the total emitted light depends on the extent of

10 molecular reorientation during the time interval between luminescence excitation and emission, which is termed the luminescence lifetime, τ . In turn, the extent of molecular reorientation depends on the luminescence lifetime and the size, shape, and environment of the reorienting molecule. Thus, luminescence polarization assays may be used to quantify binding reactions

15 and enzymatic activity, among other applications. In particular, molecules commonly rotate (or "tumble") via diffusion, with a rotational correlation time τ_{rot} that is proportional to their volume, or the cube of their radius of gyration. (This cubic dependence on radius makes polarization assays very sensitive to binding.) Thus, during their luminescence lifetime, relatively large molecules

20 will not reorient significantly, so that their total luminescence will be relatively polarized. In contrast, during the same time interval, relatively small molecules will reorient significantly, so that their total luminescence will be relatively unpolarized.

25

The relationship between polarization and intensity is expressed by the following equation:

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \quad (1)$$

Here, P is the polarization, $I_{||}$ is the intensity of luminescence polarized parallel to the polarization of the excitation light, and I_{\perp} is the intensity of luminescence polarized perpendicular to the polarization of the excitation light. P generally varies from zero to one-half for randomly oriented molecules (and zero and one for aligned molecules). If there is little rotation between excitation and emission, $I_{||}$ will be relatively large, I_{\perp} will be relatively small, and P will be close to one-half. (P may be less than one-half even if there is no rotation; for example, P will be less than one-half if the absorption and emission dipoles are not parallel.) In contrast, if there is significant rotation between absorption and emission, $I_{||}$ will be comparable to I_{\perp} , and P will be close to zero. Polarization often is reported in milli-P units ($1000 \times P$), which for randomly oriented molecules will range between 0 and 500, because P will range between zero and one-half.

Polarization also may be described using other equivalent quantities, such as anisotropy. The relationship between anisotropy and intensity is expressed by the following equation:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (2)$$

Here, r is the anisotropy. Polarization and anisotropy include the same information, although anisotropy may be more simply expressed for systems containing more than one luminophore. In the description and claims that follow, these terms may be used interchangeably, and a generic reference to one should be understood to imply a generic reference to the other.

The relationship between polarization and rotation is expressed by the Perrin equation:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) \cdot \left(1 + \frac{\tau}{\tau_{rot}}\right) \quad (3)$$

Here, P_0 is the polarization in the absence of molecular motion (intrinsic polarization), τ is the luminescence lifetime (inverse decay rate) as described above, and τ_{rot} is the rotational correlation time (inverse rotational rate) as described above.

The Perrin equation shows that luminescence polarization assays are most sensitive when the luminescence lifetime and the rotational correlation time are similar. Rotational correlation time is proportional to molecular weight, increasing by about 1 nanosecond for each 2,400 dalton increase in molecular weight (for a spherical molecule). For shorter lifetime luminophores, such as fluorescein, which has a luminescence lifetime of roughly 4 nanoseconds, luminescence polarization assays are most sensitive for molecular weights less than about 40,000 daltons. For longer lifetime probes, such as $\text{Ru}(\text{bpy})_2\text{dcbpy}$ (ruthenium 2,2'-dibipyridyl 4,4'-dicarboxyl-2,2'-bipyridine), which has a lifetime of roughly 400 nanoseconds, luminescence polarization assays are most sensitive for molecular weights between about 70,000 daltons and 4,000,000 daltons.

Luminescence polarization assays may be used in a variety of formats. In one format, the concentration of an analyte in solution can be measured by supplying a labeled tracer that competes with the analyte for a binding moiety, particularly a binding moiety larger than the labeled tracer. In this "competitive" format, the concentration of the analyte is inversely correlated with the enhancement of luminescence polarization in the light emitted by the tracer when it competitively binds the common moiety. In another format, the concentration of a target can be measured by supplying a labeled tracer that is capable of binding the target. In this case, the enhancement of polarization is a direct measure of the concentration of target. The target further may be, for example, an activated receptor, where activation can be indirectly measured by

the directly measured concentration of a generated molecule or by its binding to labeled tracer *per se*.

Appendix

The following table lists the one- and three-letter abbreviations
 5 for the twenty amino acids commonly found in biological systems.

<i>Amino acid</i>	<i>Three-letter abbreviation</i>	<i>One-letter abbreviation</i>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Although the invention has been disclosed in its preferred forms, the specific embodiments thereof as disclosed and illustrated herein are not to be
 10 considered in a limiting sense, because numerous variations are possible. As used herein, singular terms do not preclude the use of more than one of the associated element, and embodiments using more than one of a particular element are within the spirit and scope of the invention. Applicants regard the subject matter of their invention to include all novel and nonobvious

combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. No single feature, function, element or property of the disclosed embodiments is essential. The following claims define certain combinations and subcombinations of features, functions, elements, and/or properties that are regarded as novel and nonobvious. Other combinations and subcombinations may be claimed through amendment of the present claims or presentation of new claims in this or a related application. Such claims, whether they are broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of applicants' invention.

WE CLAIM:

1. A method of detecting addition or removal of a phosphate group to or from a substrate comprising
5 contacting a luminescent peptide with a binding partner that binds specifically to a phosphorylated peptide substantially without regard to the particular amino acid sequence of the peptide, wherein the peptide is a substrate for an enzyme that catalyzes addition or cleavage of a phosphate group to or from a protein, and
10 measuring luminescence polarization from the luminescent peptide, wherein the amount of measured luminescence polarization can be related to the extent of binding between the luminescent peptide and the binding partner.
2. The method of claim 1, wherein the binding partner comprises a
15 macromolecule having entrapped metal ions.
3. The method of claim 2, wherein the metal ions comprise gallium or iron.
- 20 4. The method of claim 2, wherein the macromolecule has a molecular weight between about 20 to 30 kilodaltons.
5. The method of claim 1 further comprising the step of correlating luminescence polarization with kinase activity.
25
6. The method of claim 1, wherein the peptide can be phosphorylated by one or more of the following enzymes: serine/threonine kinase, threonine/tyrosine kinase, and tyrosine kinase.

7. The method of claim 1 further comprising the step of correlating luminescence polarization with phosphatase activity.
8. The method of claim 1, wherein the protein and the peptide are
5 the same.
9. The method of claim 1, wherein the protein and the peptide are different.
- 10 10. The method of claim 1 further comprising the step of providing at least one phosphate group on the luminescent peptide, and competing with the luminescent peptide by catalyzing formation of unlabelled phosphorylated protein.
- 15 11. The method of claim 1, wherein the substrate has serine and threonine residues.
12. The method of claim 1, wherein the peptide is amidated on one end.

13. The method of claim 1, wherein the peptide is selected from the group consisting of

ApYTGLSTRNQETpYATH-NH₂,
pYpYpYIE-NH₂,
5 GpYNELNLGRREEpYDVL-NH₂,
EKRPpSRSKYL,
EKRPpSRpSYL,
EKRPpSRpSYL,
KRREILSRRpSYRK,
10 KHFPQFpSYSAS,
pSPELERLIQC,
GSPSVRCSpSMpS,
RSRHSpSYPAGT,
LpTPLK,
15 FpTPLQ,
RKRpTLRRL,
LRRApSLG,
KKLNRTLpSVASL,
RPRAApTF-NH₂ and
20 LRRApSLG-NH₂.

14. The method of claim 1 further comprising illuminating the sample with polarized light from a high color temperature continuous light source

15. A method of detecting addition or removal of a phosphate group to or from a substrate comprising

contacting a luminescent peptide with a binding partner that binds specifically to the peptide only if the peptide is phosphorylated, or only if the peptide is not phosphorylated, wherein the peptide is a substrate for an enzyme
5 that catalyzes addition or cleavage of a phosphate group to or from a protein, the peptide having at least one amino acid selected from the group consisting of serine and threonine, where the serine or threonine is either near a sequence that is enriched with basic amino acids, or followed in the C-terminal direction
10 by a proline residue, and

measuring luminescence polarization from the luminescent peptide, wherein the amount of measured luminescence polarization can be related to the extent of binding between the luminescent peptide and the binding partner.

15 16. The method of claim 15 further comprising the step of correlating luminescence polarization with kinase activity.

17. The method of claim 15 further comprising the step of correlating luminescence polarization with phosphatase activity.

20

18. The method of claim 15, wherein the peptide has fewer than about 15 amino acids.

19. The method of claim 15, wherein the protein and the peptide are
25 the same.

20. The method of claim 15, wherein the protein and the peptide are different.

21. The method of claim 15 further comprising the step of providing at least one phosphate group on the luminescent peptide, and competing with the luminescent peptide by catalyzing formation of unlabelled phosphorylated protein.

5

22. The method of claim 15, wherein the binding partner binds specifically to a phosphorylated protein substantially without regard to the particular amino acid sequence of the protein.

10

23. The method of claim 22, wherein the binding partner comprises a macromolecule having entrapped metal ions.

24. The method of claim 23, wherein the metal ions comprise gallium or iron.

15

25. The method of claim 15, wherein the substrate has serine and threonine residues.

26. The method of claim 15, wherein the peptide is amidated on one end.

20

27. The method of claim 15, wherein the basic amino acids are selected from the group consisting of lysine, arginine, and histidine.

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28. The method of claim 15, wherein the serine or threonine is followed within three amino acids in the C-terminal direction by a basic amino acid.

29. The method of claim 15, wherein the serine or threonine is followed immediately in the C-terminal direction by a proline residue.

30. The method of claim 15, wherein the peptide is selected from the
5 group consisting of

EKR_PpSR_SSKYL,
EKR_PPSQR_PSYL,
EKR_PpSR_PSYL,
KRREILSRR_PpSYRK,
10 KHFPQF_PSY_SSAS,
pSPELERLIIQC,
GSPSVRC_SpSM_PS,
RSRH_SpSY_PPAGT,
L_PTPLK,
15 F_PTPLQ,
RKR_PTLRRL,
LRR_ApSLG,
KKLNRTL_PSVASL,
RPRAA_PTF-NH₂, and
20 LRR_ApSLG-NH₂.

31. The method of claim 15 further comprising illuminating the sample with polarized light from a high color temperature continuous light source

32. A method of detecting addition or removal of a phosphate group to or from a substrate comprising

contacting a luminescent peptide with a binding partner that binds specifically to a phosphorylated peptide substantially without regard to the particular amino acid sequence of the peptide, wherein the peptide is a substrate for an enzyme that catalyzes addition or cleavage of a phosphate group to or from a protein, and

measuring luminescence resonance energy transfer (FRET), and relating FRET to the extent of binding between the luminescent peptide and the binding partner.

33. The method of claim 32 further comprising the step of correlating FRET with kinase activity.

34. The method of claim 33, wherein the peptide can be phosphorylated by one or more of the following enzymes: serine/threonine kinase, threonine/tyrosine kinase, and tyrosine kinase.

35. The method of claim 32 further comprising the step of correlating FRET with phosphatase activity.

36. The method of claim 32, wherein the protein and the peptide are the same.

37. The method of claim 32, wherein the protein and the peptide are different.

38. The method of claim 32 further comprising the step of providing at least one phosphate group on the luminescent peptide, and competing with the luminescent peptide by catalyzing formation of unlabelled phosphorylated protein.

5

39. The method of claim 32, wherein the substrate has at least one serine or threonine residue.

40. The method of claim 32, wherein the binding partner comprises a
10 macromolecule having entrapped metal ions.

41. The method of claim 40, wherein the metal ions comprise gallium or iron.

15 42. The method of claim 40, wherein the macromolecule also comprises ruthenium.

43. The method of claim 40, wherein the macromolecule has a molecular weight between about 20 to 30 kilodaltons.

44. A kinase substrate comprising
a luminophore, and
a peptide sequence selected from the group consisting of
- 5 AYTGLSTRNQETYATH-NH₂,
 YYYIE-NH₂,
 GYNELNLGRREEYDVL-NH₂,
 EKRPSRSKYL
 EKRPSQRSYL,
 EKRPSRSYL,
10 KRREILSRRPSYRK,
 KHFPQFSYSAS,
 SPELERLIIQC,
 GSPSVRCSSMS,
 RSRHSSYPAGT,
15 LTPLK,
 FTPLQ,
 RKRTLRL,
 LRRASLG,
 KKLNRTLSTVASL,
20 RPRAATF-NH₂, and
 LRRASLG-NH₂.

45. The substrate of claim 44 further comprising at least one
phosphate substituent that can be cleaved by a phosphatase enzyme.

46. The substrate of claim 44, wherein the substrate can be phosphorylated by tyrosine kinase, the peptide having an amino acid sequence selected from the group consisting of

AYTGLSTRNQETYATH-NH₂,

5 YYYIE-NH₂, and

GYNELNLGRREEYDVL-NH₂.

47. The substrate of claim 44, wherein the substrate can be phosphorylated by a serine/threonine kinase, the peptide being selected from
10 the group consisting of

EKRPSRSKYL,

EKRPSQRSYL,

EKRPSRSYL,

KRREILSRPSYRK,

15 KHFPQFSYSAS,

SPELERLIIQC,

GSPSVRCSSMS,

RSRHSSYPAGT,

LTPLK,

20 FTPLQ,

RKRTLRL,

LRRASLG,

KKLNRTL SVASL,

RPRAATF-NH₂, and

25 LRRASLG-NH₂.

48. A composition comprising at least one peptide selected from the following group

5 EKRpSRSKYL,
EKRPSQRpSYL,
EKRpSRpSYL,
KRREILSRPpSYRK,
KHFPQFpSYSAS,
pSPELERLIIQC,
GSPSVRCSpSMpS,
10 RSRHSpSYPAGT,
LpTPLK,
FpTPLQ,
RKRpTLRRL,
LRRApSLG,
15 KKLNRTLpSVASL,
RPRAApTF-NH₂, and
LRRApSLG-NH₂.

49. The composition of claim 48 further comprising a luminophore
20 associated with at least one of the peptides.

50. A composition comprising a binding partner that binds
specifically to a peptide having at least one serine or threonine, where the
serine or threonine is either near a sequence that is enriched with basic amino
25 acids, or followed in the C-terminal direction by a proline residue.

51. The composition of claim 50, wherein at least one of said serine
or threonine is phosphorylated.

52. The composition of claim 50, the binding partner binds specifically to at least one peptide selected from the following group

5 EKR_pSRSKYL,
EKRPSQR_pSYL,
EKR_pSR_pSYL,
KRREILSR_pSYRK,
KHFPQF_pSYSAS,
pSPELERLIIQC,
GSPSVRCSpSM_pS,
10 RSRHSpSYAGT,
LpTPLK,
FpTPLQ,
RKR_pTLRRL,
LRRApSLG,
15 KKLNRTL_pSVASL,
RPRAApTF-NH₂, and
LRRApSLG-NH₂.

53. The composition of claim 50, 51, or 52 further comprising a
20 luminophore associated with at least one of the specific binding partners.

54. A compound of the formula
FLApYTGLSTRNQET_pYATH-NH₂,
FLpYpYpYIE-NH₂, or
25 FLGpYNELNLGRREE_pYDVL-NH₂,

wherein FL represents a luminophore and p represents a phosphate group.

55. The compound of claim 54, wherein said luminophore is fluorescein or a fluorescein derivative.

56. A method to determine a tyrosine kinase activity or the concentration of a protein containing phosphorylated tyrosine which method comprises

contacting a sample to be tested with the compound of claim 54 and with the opposite member of a specific binding pair comprising said compound,

10 illuminating the sample with polarized light comprising a wavelength to effect excitation of said luminophore so as to generate polarized emitted light, and

measuring the degree of polarization of said emitted light, wherein the measured degree of polarization of said emitted light is inversely correlated with the activity or concentration of tyrosine kinase or of the protein containing phosphorylated tyrosine in said sample.

15

57. A method to assess the ability of a candidate compound to enhance or inhibit phosphorylation of tyrosine in a substrate protein which method comprises

performing the method of claim 56 in the presence and absence of said
5 candidate compound, and

determining the degree of polarization in the presence as compared to the absence of said candidate compound, whereby an increase in the degree of measured polarization in the presence as opposed to the absence of said candidate compound identifies said compound as an inhibitor of said
10 phosphorylation, and whereby a decrease in the degree of polarization in the presence as compared to the absence of said compound identifies said compound as an enhancer of said phosphorylation.

58. A method for determining tyrosine kinase activity, the method
15 comprising:

performing a tyrosine kinase reaction in a solution to produce a phosphorylated product,

adding a mixture of stop solution, anti-phosphotyrosine antibody, and luminescently labeled tracer, wherein the stop solution stops the kinase reaction
20 and the luminescently labeled tracer competes with the phosphorylated product for binding to the anti-phosphotyrosine antibody, and

measuring the luminescence polarization of the solution.

59. A method as in claim 58 conducted in a microplate well.

25

60. A method as in claim 58, wherein the stop solution comprises a chelator.

61. A method to determine the presence and amount of a posttranslationally modified protein or peptide,

which method comprises contacting a sample containing said posttranslationally modified protein or peptide with a luminescent tracer and a
5 specific binding partner, wherein said tracer and said posttranslationally modified protein or peptide compete for binding to said partner, and wherein, when said tracer is bound to said partner, its luminescence polarization is changed from that of the tracer in unbound form,

followed by measuring the luminescence polarization in the sample,
10 whereby a change in the luminescence polarization to more closely approximate that of the tracer in unbound form is a measure of the presence or amount of said posttranslationally modified protein or peptide.

62. The method of claim 61, wherein the posttranslational
15 modification results in a posttranslationally modified substrate from an enzyme catalyzed reaction of a protein or peptide substrate.

63. The method of claim 62, wherein the posttranslationally modified
20 substrate is a peptide or protein phosphorylated at one or more amino acid residues.

64. The method of claim 63, wherein the phosphorylated residue in the peptide or protein is tyrosine.

25 65. The method of claim 64, wherein the tracer is a peptide containing a phosphorylated tyrosine which mimics the phosphorylation site of the substrate.

66. The method of claim 62, wherein the amount of posttranslationally modified substrate results in the sample from the enzyme catalyzed reaction of a protein or peptide substrate and is a measure of the concentration or activity of the enzyme.

5

67. The method of claim 66, wherein said sample further includes a candidate inhibitor or stimulator of the enzyme catalyzed reaction and wherein the rate of change in luminescence polarization is increased in the presence of a stimulator and diminished in the presence of an inhibitor.

10

68. A method to identify a candidate substance, which substance will be effective in modulating activity of a target enzyme or enzymatically active target receptor, which method comprises subjecting the candidate substance as a sample to the method of claim 66, wherein an increase or decrease in the rate of change in luminescence polarization indicates the ability of the candidate substance to modulate the target.

15

69. The method of claim 62, wherein said enzyme catalyzed reaction is that catalyzed by the insulin receptor cytoplasmic kinase domain (IR-CKD) or IGF-1r cytoplasmic kinase domain (IGF-1r-CKD).

20

70. The method of claim 69, wherein the modified form of the substrate is the phosphorylated form of biotin-EGPWLEEEEEAYGWMDF-amide (KS2-peptide), DYMTMQIG (D-peptide), or SRGDYMTMQIG (S-peptide).

25

71. A peptide selected from the group consisting of the phosphorylated forms of: KS2, D-peptide, S-peptide, and N-acetyl-O-phosphotyramine.

72. A method to map a protein for the location of modification sites which method comprises

- a) digesting said protein with a protease, to obtain fragments,
- b) separating the fragments,
- 5 c) treating the fragments with a luminescence polarization mixture comprising a tracer and an opposing specific binding pair member for which tracer and any modified fragments compete, and
- d) detecting a change in luminescence polarization as an indication of a modified fragment.

10

73. A method to determine the presence and amount of a posttranslationally modified protein or peptide comprising,

- contacting a sample containing said posttranslationally modified protein or peptide with a luminescence tracer and a specific binding partner, wherein
- 15 said tracer and said posttranslationally modified protein or peptide compete for binding to said partner, and wherein, when said tracer is bound to said partner, its luminescence polarization is changed from that of the tracer in unbound form,

- followed by measuring the luminescence polarization in the sample,
- 20 whereby a change in the luminescence polarization to more closely approximate that of the tracer in unbound form is a measure of the presence or amount of said posttranslationally modified protein or peptide,

- wherein the posttranslational modification results in a posttranslationally modified substrate from an enzyme catalyzed reaction of a protein or peptide
- 25 substrate that is catalyzed by the insulin receptor cytoplasmic kinase domain (IR-CKD) or IGF-1r cytoplasmic kinase domain (IGF-1r-CKD), and wherein the tracer is a fluorescein conjugate of the peptide GEEGYMPMGK or N-acetyl-O-phosphotyramine.

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Fig. 1

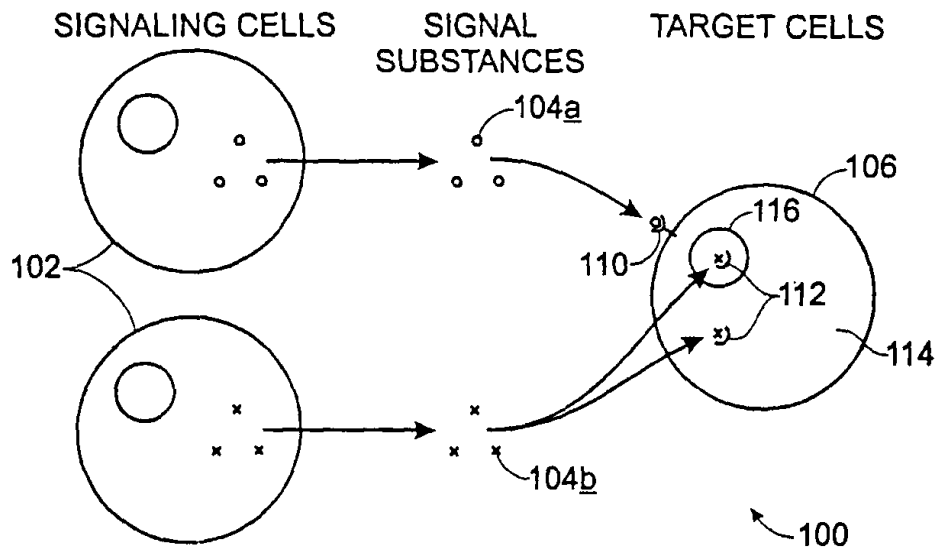


Fig. 2

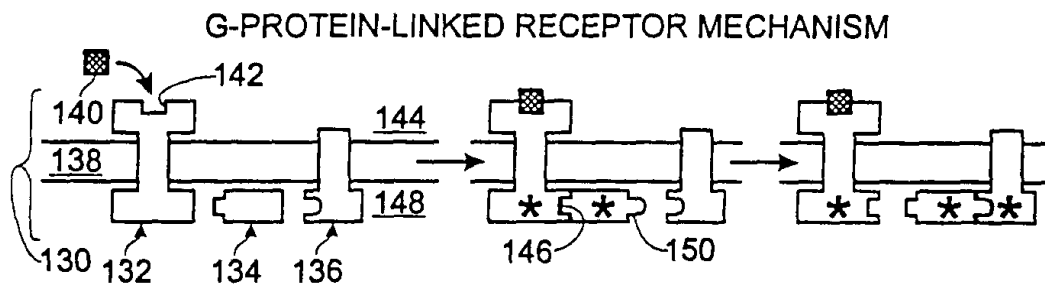
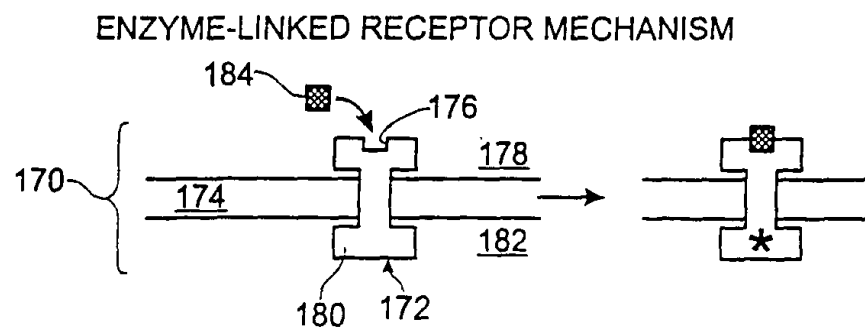
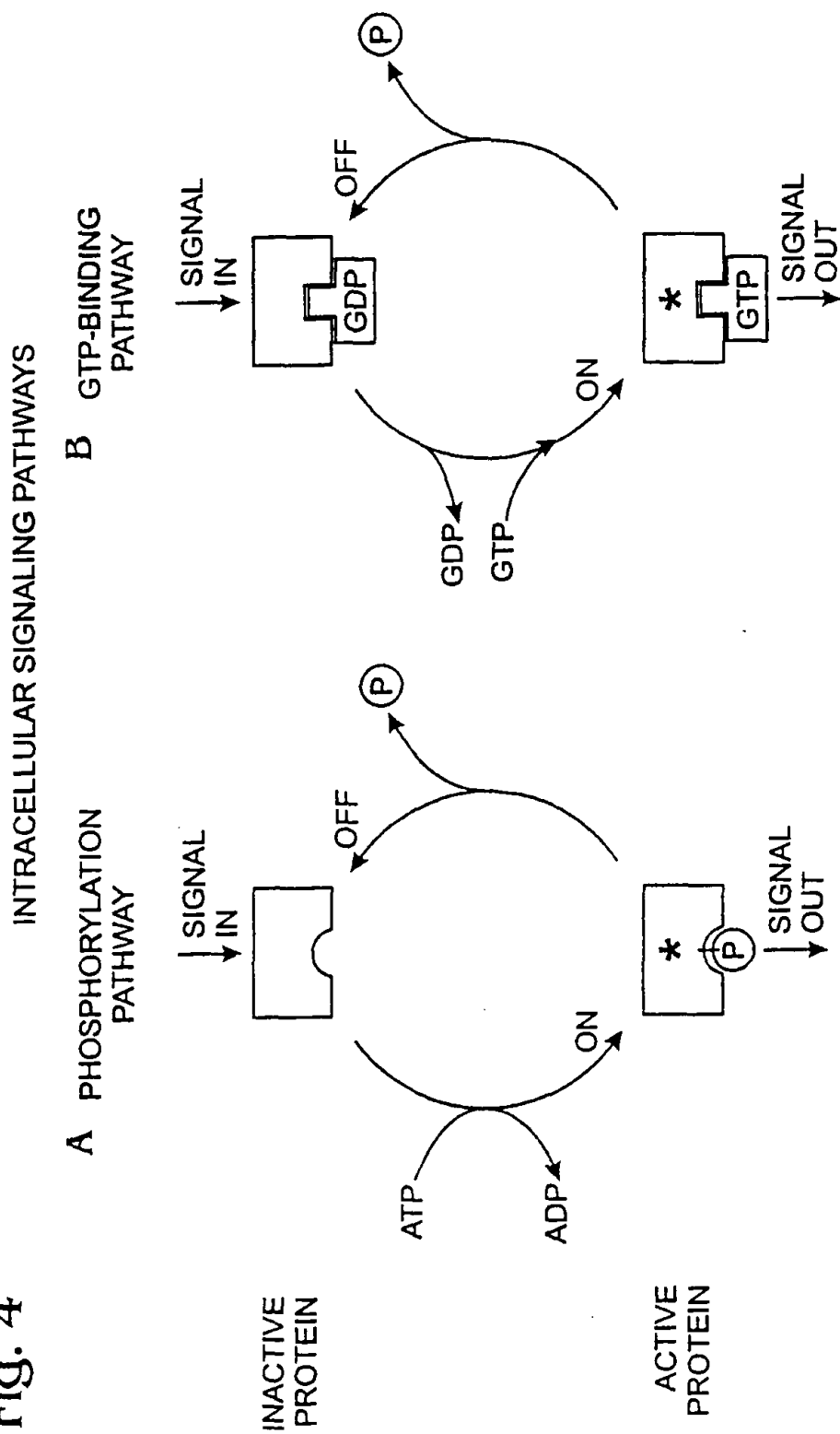


Fig. 3



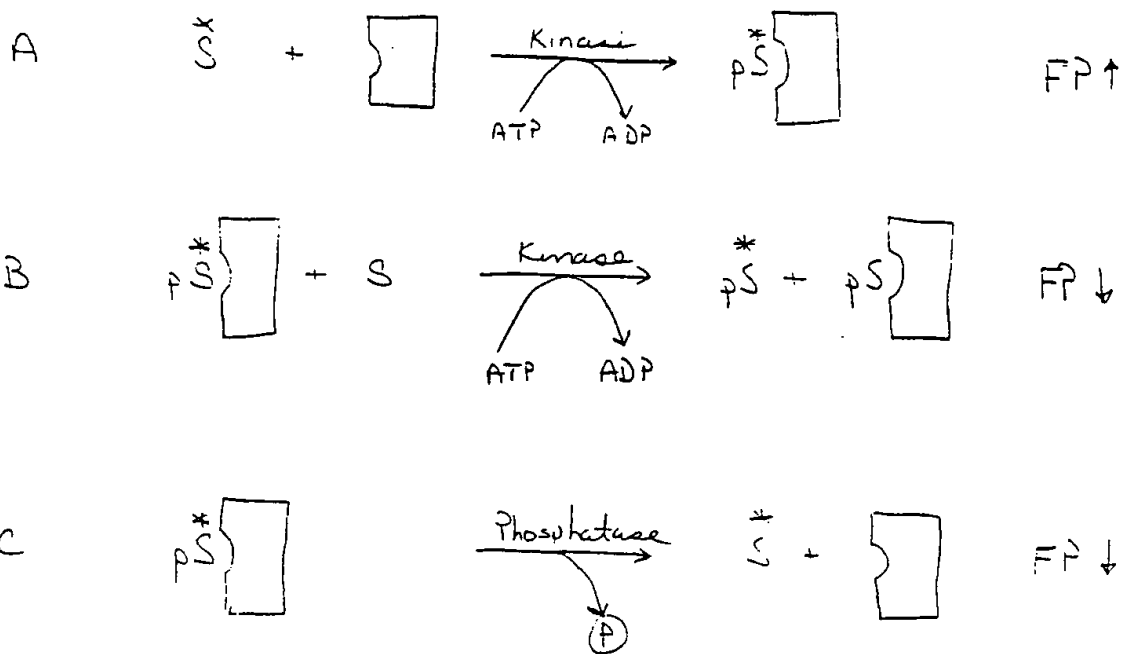
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Fig. 4



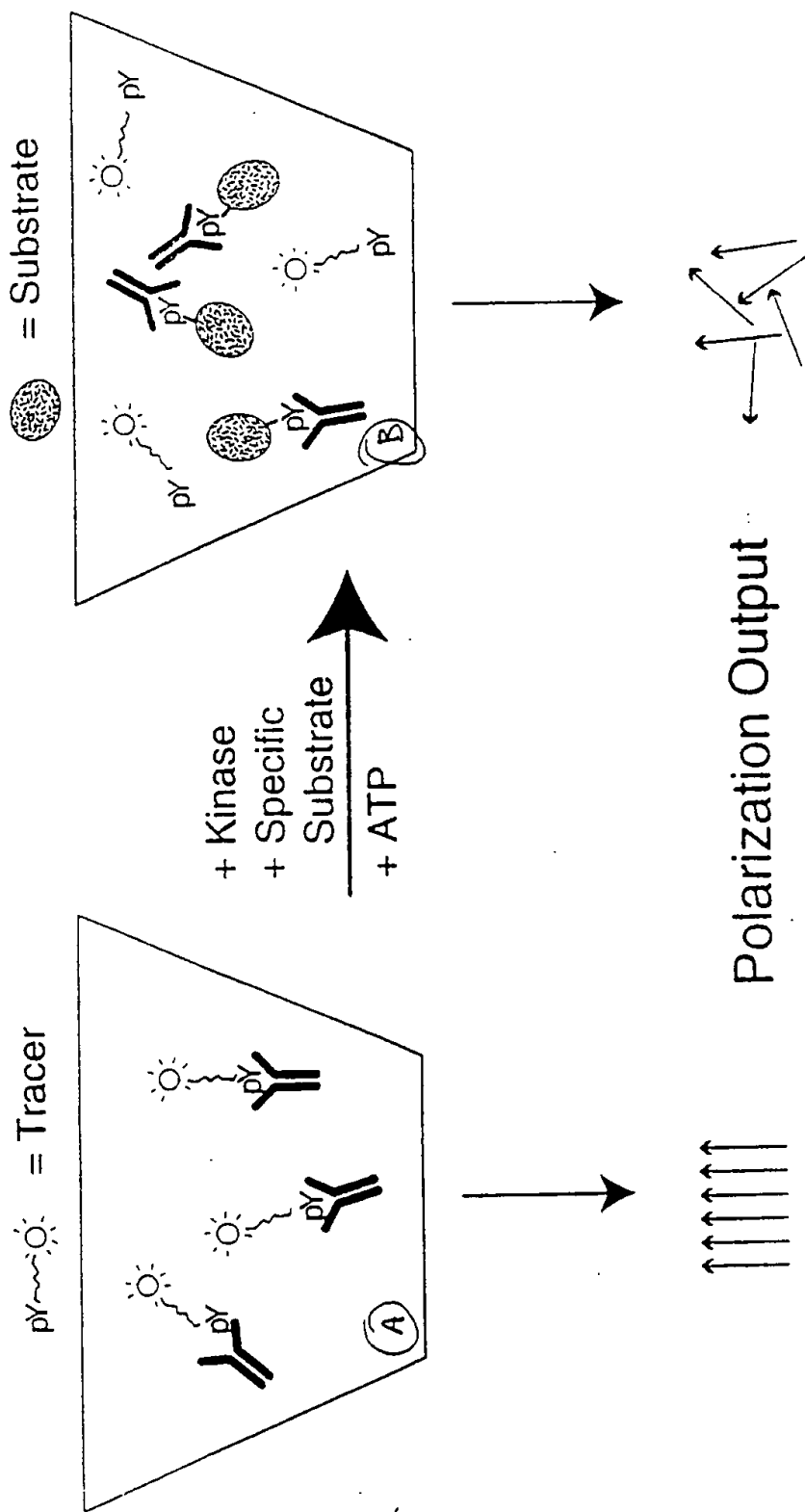
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Fig. 5



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Fluorescence Polarization Displacement Assay



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Fig. 7

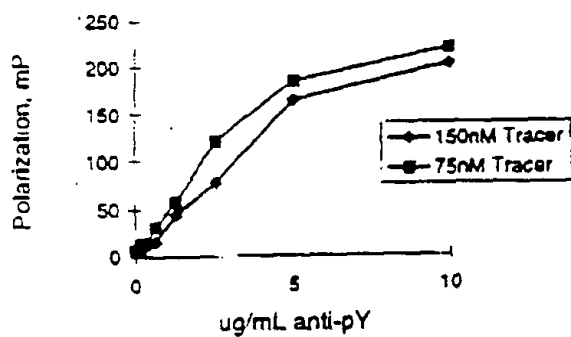
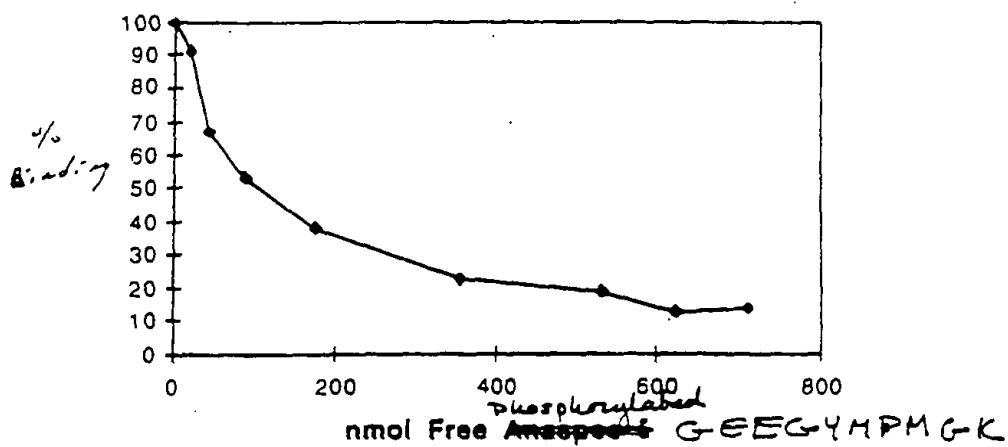


Fig. 8



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Fig. 9

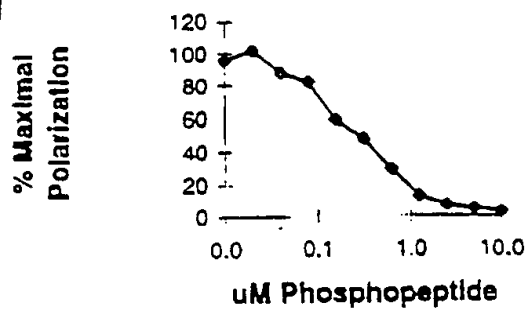
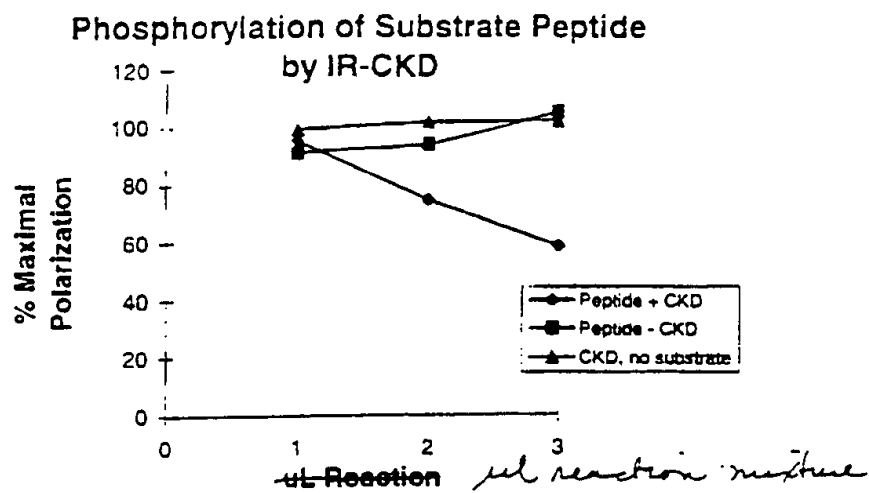


Fig. 10



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Fig 11

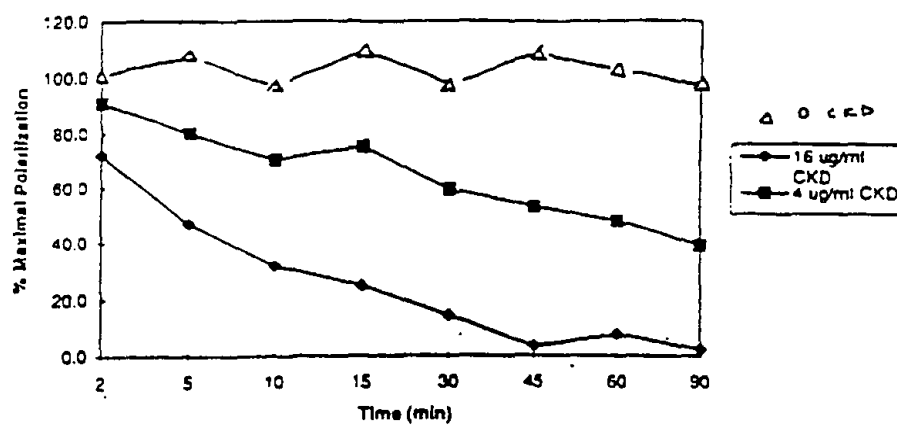
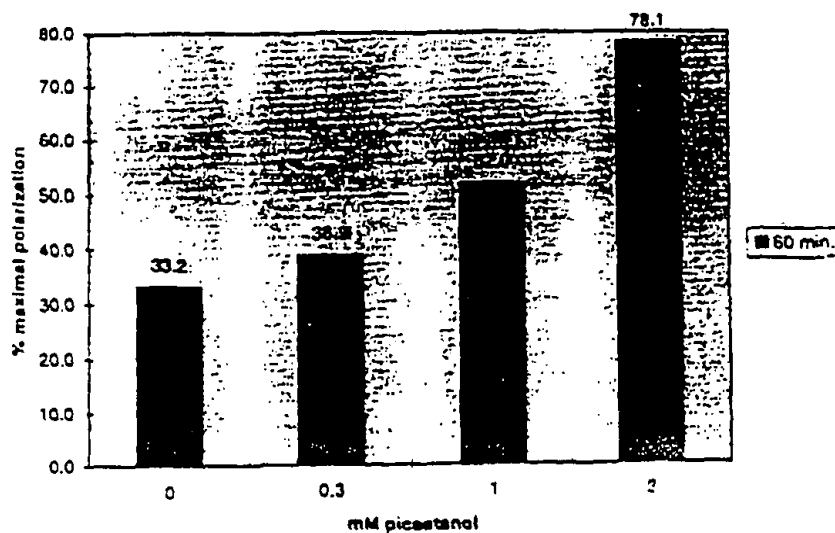
Time course for the effect of presence of IR-CKD in
Phosphorylation of "S"-Peptide

Fig. 12

3440671 inhibitory effect of piceatanol on IR-CKD in
phosphorylation of "S" peptide

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FIGURE 13 SCREEN FOR AGONIST ACTIVITY USING IR-CKD

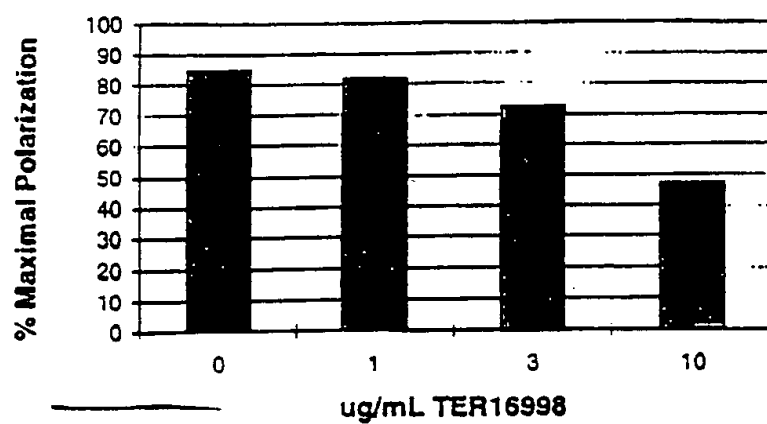
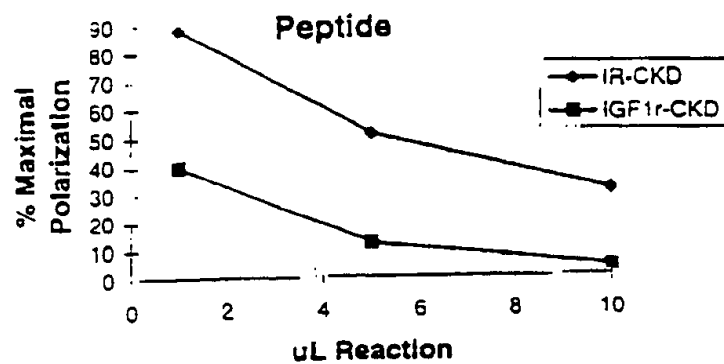


Fig. 14



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Fig. 15

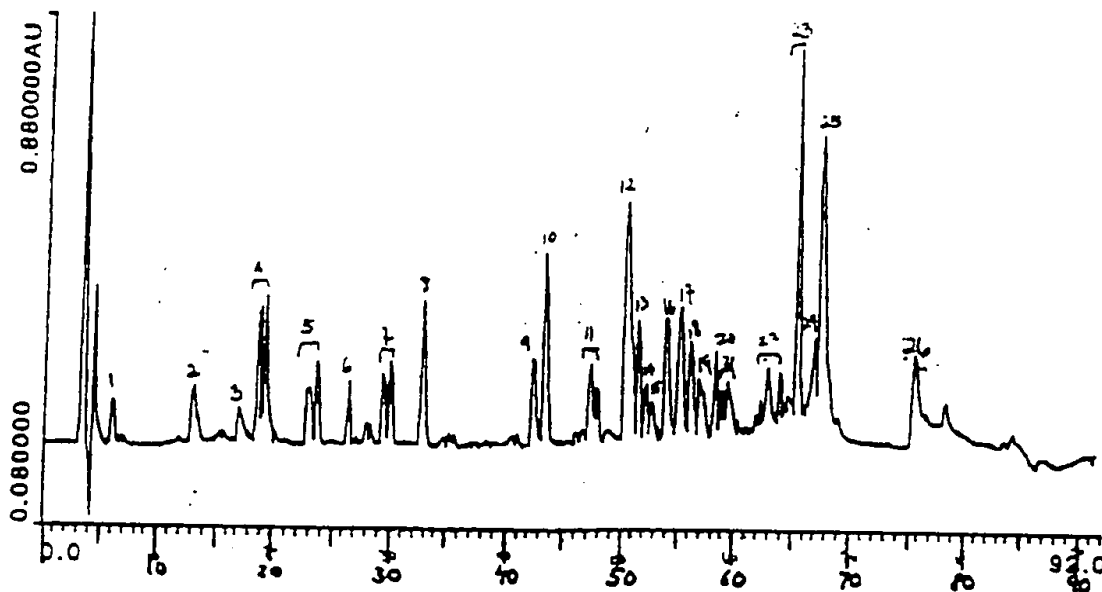
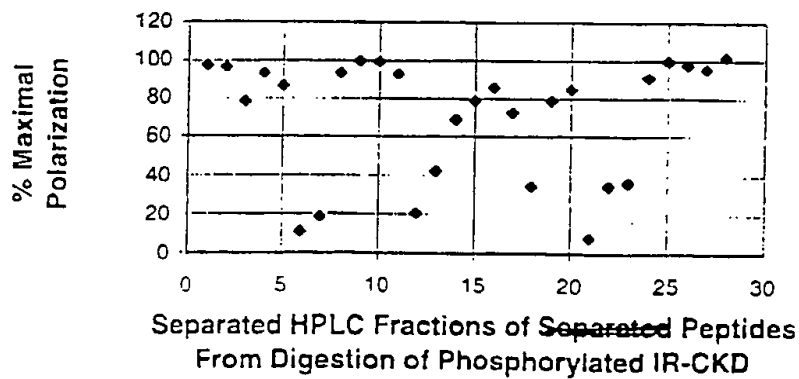
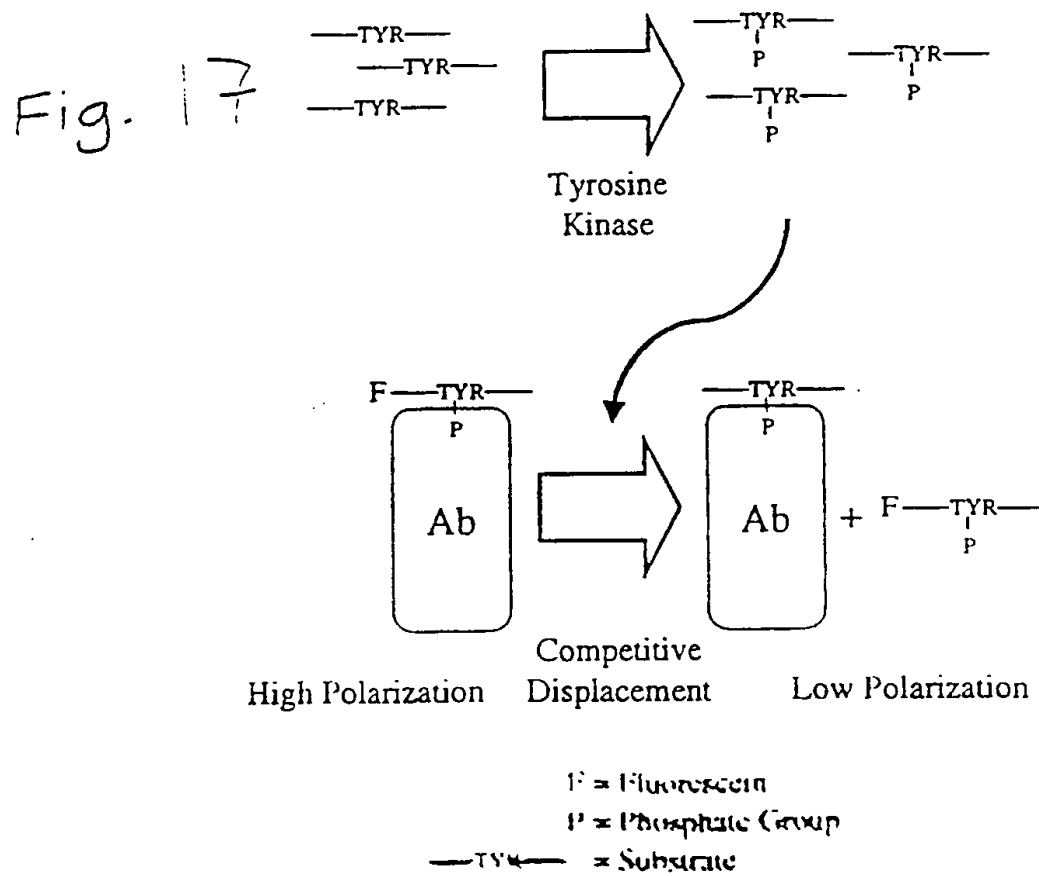


Fig. 16

**pY Peptide Mapping: Phosphorylation
Detection by Fluorescence Polarization**



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Fig. 18

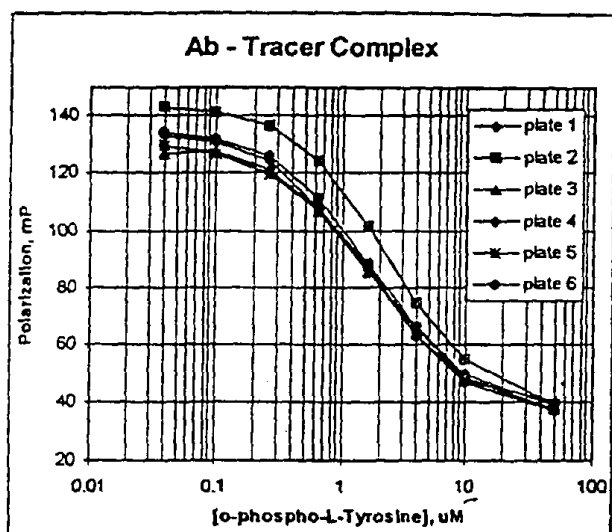
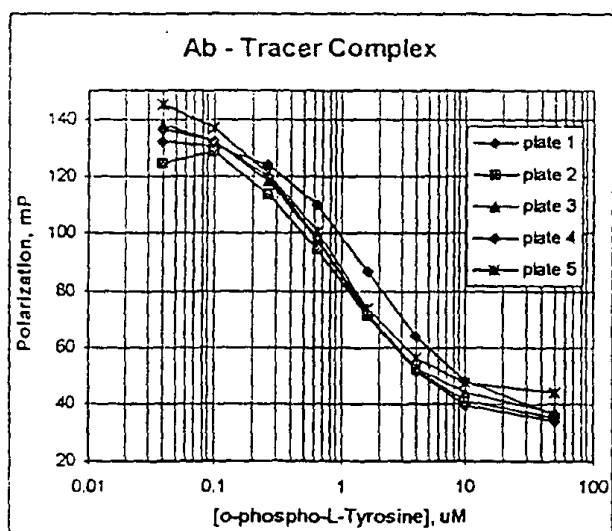


Fig. 19



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Fig. 20

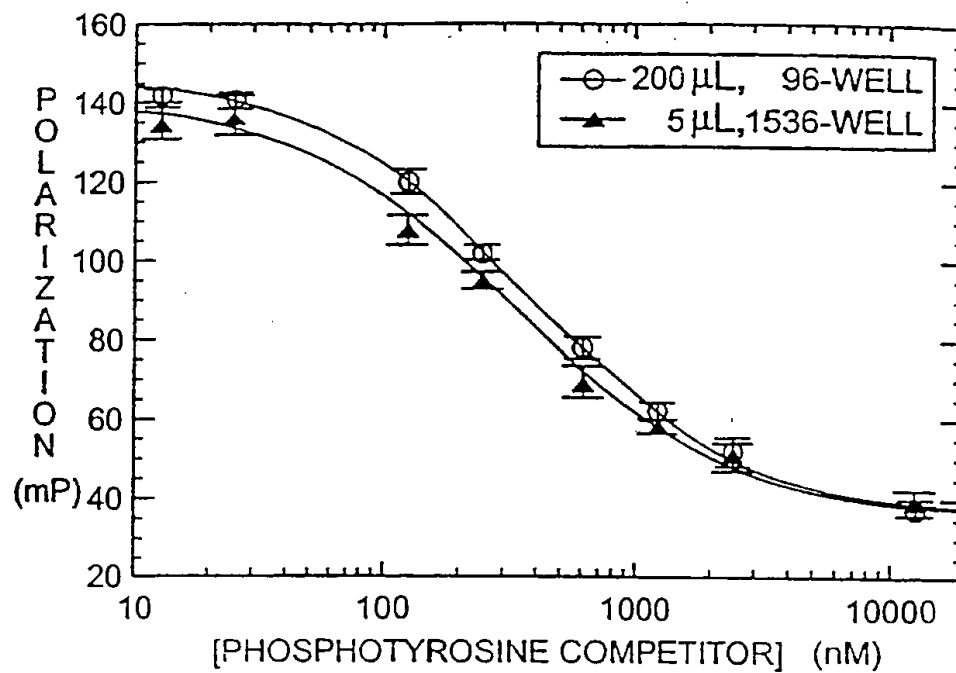
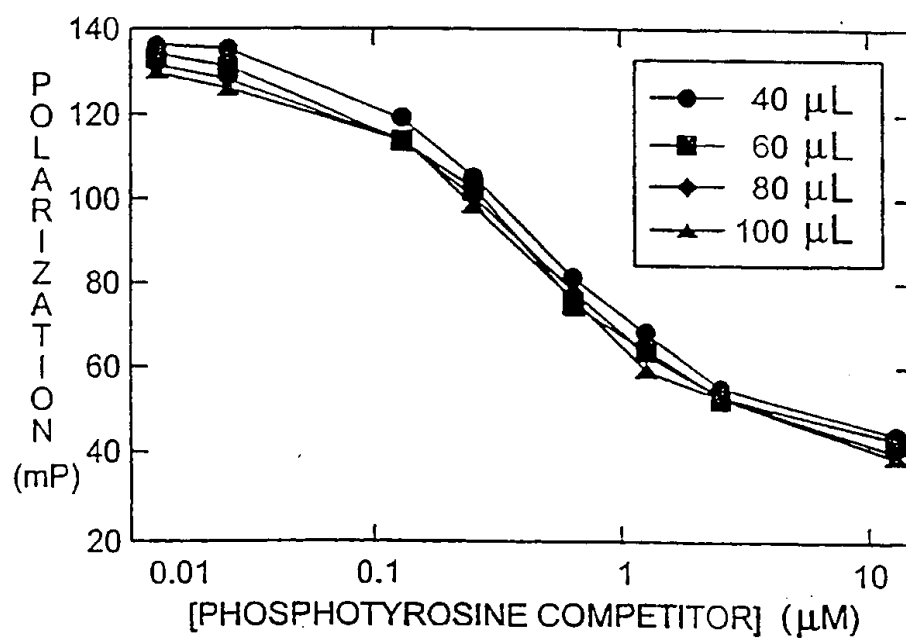
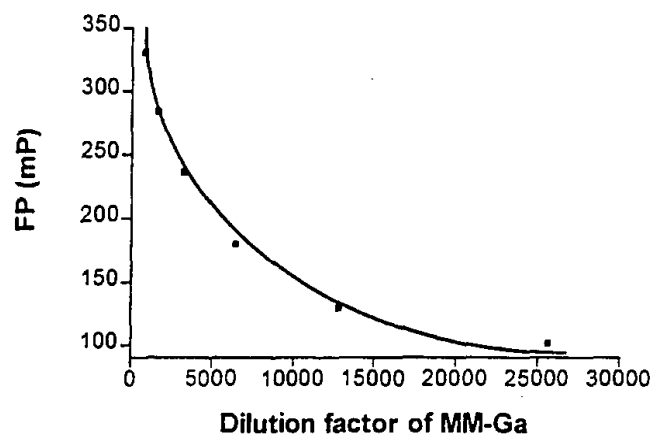
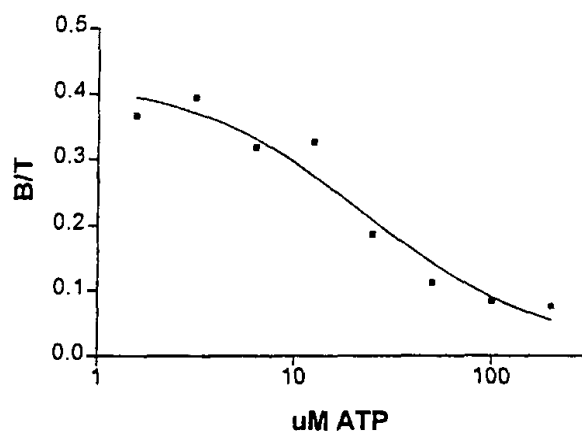


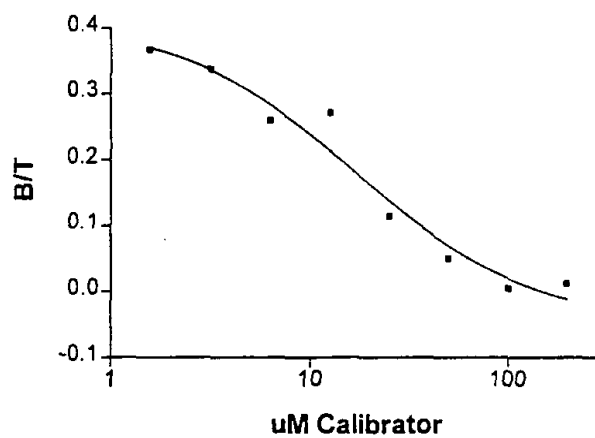
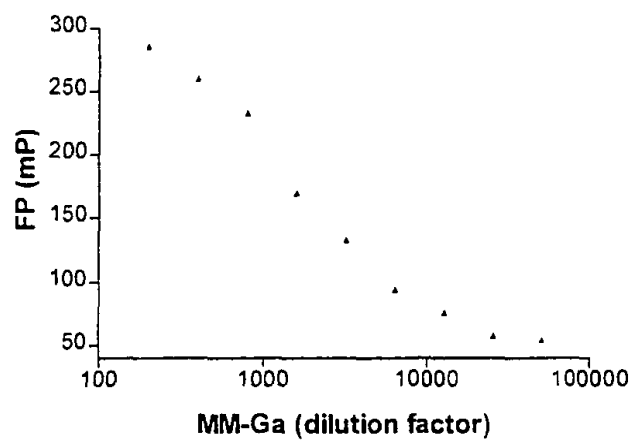
Fig. 21



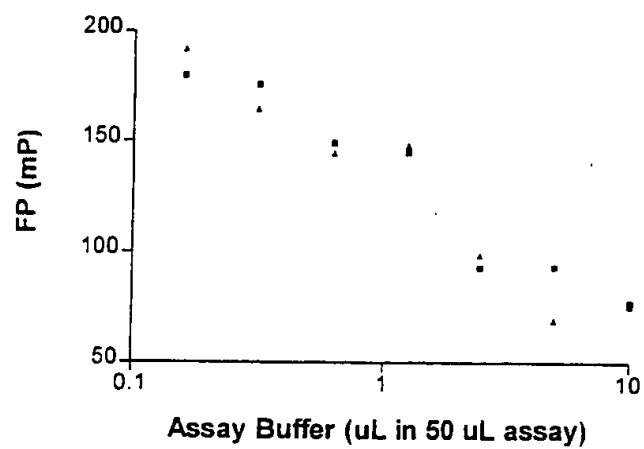
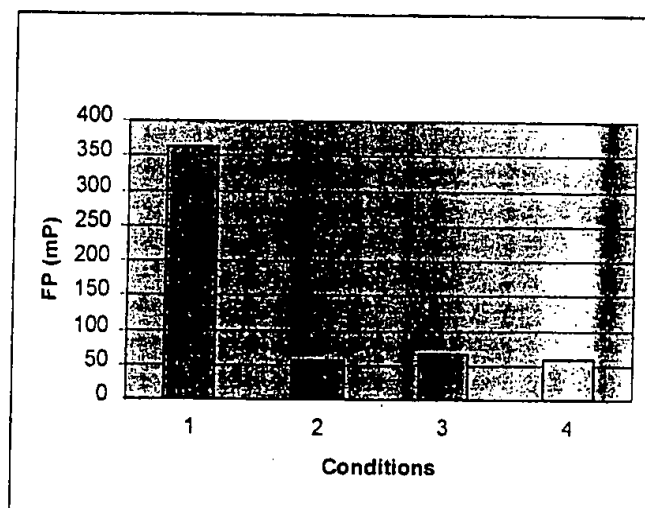
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Fig. 22**Fig. 23**

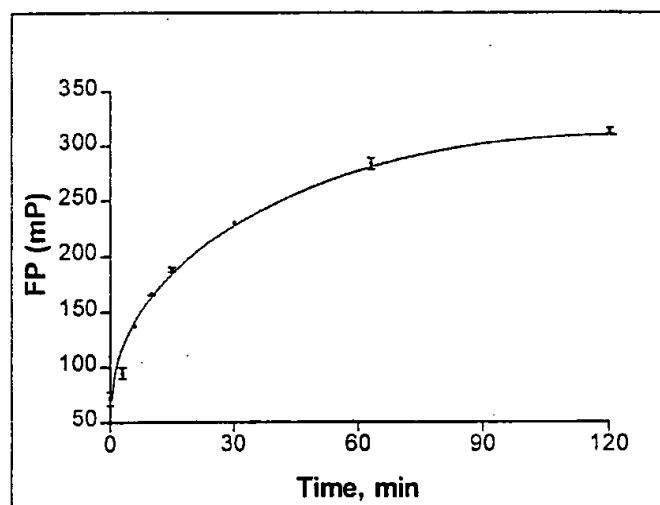
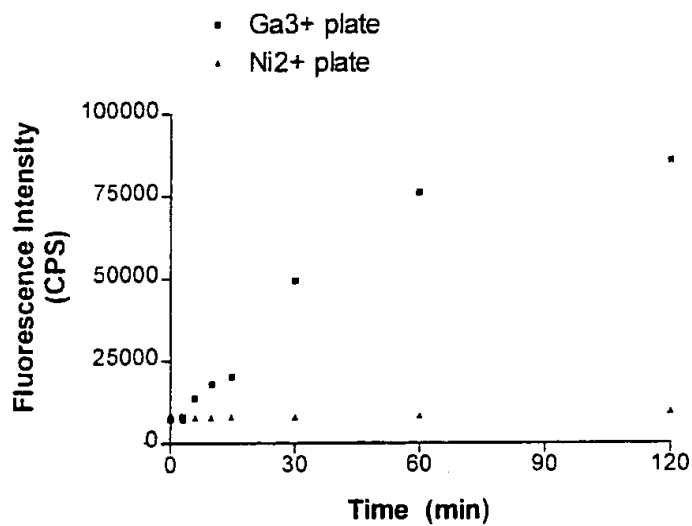
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Fig. 24**Fig. 25**

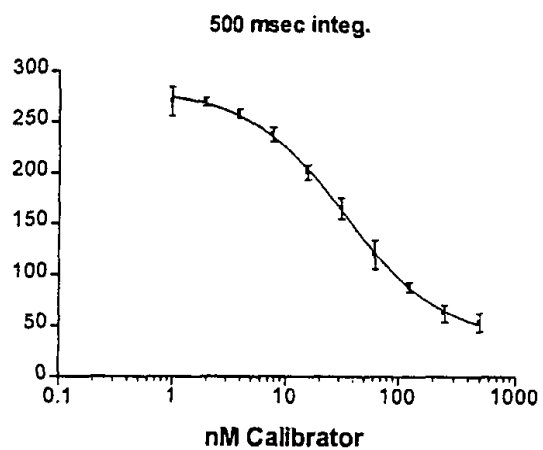
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Fig. 26**Fig. 27**

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Fig. 28**Fig. 29**

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Fig. 30**A****B**